

University of Alberta

Characterization of *cis* and *trans* silencing of a *P{lacW}* transgene on
chromosome 4 of *Drosophila melanogaster*

by



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Abstract

$P(lacW)cf^{Dplac}$ is a $P(lacW)$ transgene inserted about 3kb distal to the *ci* transcription unit on chromosome 4 in *Drosophila melanogaster*. Its *white* mini-gene marker produces a uniformly red pigmented eye. $P(lacW)cf^{E1}$ and $P(lacW)cf^{E2}$ are independent spontaneous mutations derived from the $P(lacW)cf^{Dplac}$ allele. Both have acquired a *gypsy* transposable element insertion near the $P(lacW)$ insertion, which may be responsible for the *cis*-silencing of the *white* mini-gene to give a variegated-eye-phenotype. My results support this model for the *cis*-silencing. $P(lacW)cf^{E1}$ and $P(lacW)cf^{E2}$ can also *trans*-silence the *white*⁺ expression from a $P(lacW)cf^{Dplac}$ homolog. I show that excising $P(lacW)$ from $P(lacW)cf^{E1}$ and $P(lacW)cf^{E2}$ does not remove their ability to *trans*-silence, but the degree of this *trans*-silencing does depend on the extent of $P(lacW)$ retained by the $P(lacW)cf^E$ alleles. Characterization of the *cis*- and *trans*-silencing in this system explores the relationship between transposons, chromosome structure, and chromatin proteins in gene silencing.

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List of Abbreviations

μ l	micro liter
$^{\circ}$ C	degrees for temperature
2-M1021.R	<i>P{hsp26-pt-T}cf^{2-M1021.R}</i>
<i>bw</i>	the eye color gene <i>brown</i>
<i>bw^D</i>	the <i>brown-Dominant</i> allele
<i>bw^V</i>	alleles caused by mobilizing <i>bw</i> next to heterochromatin
CHCl ₃	chloroform
<i>ci</i>	the <i>cubitus interruptus</i> gene
dATP	deoxyadenosine triphosphate
dCTP	deoxycytidine triphosphate
dGTP	deoxyguanosine triphosphate
DNA	DeoxyriboNucleicAcid
dNTP	deoxynucleoside triphosphate
dp	a mutant dumpy wing phenotype (no italics)
dp ⁺	a wild type dumpy wing phenotype (no italics)
dTTP	deoxythymidine triphosphate
<i>e</i>	a mutant ebony body phenotype (no italics)
<i>E(var)</i>	enhancers of position effect variegation
<i>e⁺</i>	a wild type ebony body phenotype (no italics)
<i>E1</i>	<i>P{lacW}cf^{E1}</i>
<i>E1R#</i>	<i>P{lacW}cf^{E1}</i> derivatives with a red eye phenotype (R=red).
<i>E1W#</i>	<i>P{lacW}cf^{E1}</i> derivatives with a white eye phenotype (W=white).
<i>E2</i>	<i>P{lacW}cf^{E2}</i>
<i>E2R#</i>	<i>P{lacW}cf^{E2}</i> derivatives with a red eye phenotype (R=red).
<i>E2W#</i>	<i>P{lacW}cf^{E2}</i> derivatives with a white eye phenotype (W=white).
EDTA	ethylene diamine tetra-acetic acid
<i>ey^D</i>	<i>Dp(2;4)ey^D, ey^DAlp^{ey^D}</i>
g	grams
H ₂ O	water
H ₂ O ₂	hydrogen peroxide
H3	one of the proteins within the histone octamer
H3-mK9	methylation at lysine 9 on histone H3
HCl	hydrochloric acid

HEPES	N(2-hydroxyethyl)piperazine- <i>N</i> -(2-ethanesulphonic acid) (buffer)
HMTase	histone methyltransferase
HP1	heterochromatin protein 1
hPEV	heterochromatic position effect variegation
jpeg	pictures are all saved as .jpeg files, and jpeg stands for Joint Photographic Experts Group and is suited for large quality photographic images
kbp	kilo base pairs
M	molar
<i>M^{62F}</i>	<i>DI(4)M101-62F</i>
Mbp	mega base pairs
MgCl ₂	magnesium chloride
min	minute
ml	milliliter
<i>mod(mdg4)</i>	the <i>modifier of mdg4</i> (<i>mod(mdg4)</i>) gene
Na ₃ PO ₄	sodium phosphate
NaCl	sodium chloride
NaOH	sodium hydroxide
NTP	nucleoside triphosphate
OLB	oligolabelling buffer
<i>P</i>	a type of transposable element
<i>P(lacW)</i>	a <i>P</i> construct with a <i>lacZ</i> reporter gene and a <i>white</i> mini-gene
marker	
<i>Pci</i>	<i>P(lacW)ci^{Opac}</i>
PCR	polymerase chain reaction
pd(N) ₆	Pharmacia (#27-2166) random hexamers
PDS	<i>P</i> element dependent silencing
PEV	position effect variegation
pH	the power of hydrogen
<i>P-lacZ</i>	<i>P</i> constructs that have <i>lacZ</i> gene fused to the <i>P</i> promoter
RdRP	RNA dependent RNA polymerase
RISC	RNA-induced silencing complex
RNA	RiboNucleicAcid
RNAi	RNA interference
<i>S. pombe</i>	<i>Schizosaccharomyces pombe</i>

SDS	sodium dodecyl sulfate
SET	a motif which was first identified as a C-terminal region of homology between the SU(VAR)3-9, ENHANCER of ZESTE and TRITHORAX proteins
siRNA	small interfering RNA
SSC	sodium citrate buffer
<i>su(Hw)</i>	the <i>suppressor of Hairy-wing</i> gene
<i>Su(var)</i>	suppressors of position effect variegation
<i>Su(var)2-5</i>	gene encoding HP1
SU(VAR)2-5	HP1, heterochromatin protein 1
<i>Su(var)3-7</i>	gene encoding the zinc finger protein
SU(VAR)3-7	the zinc finger protein
<i>Su(var)3-9</i>	gene encoding the histone methyltransferase
SU(VAR)3-9	the histone methyltransferase
TE	Tris-EDTA
<i>w</i>	a null mutant <i>white</i> gene genotype (italics)
<i>w</i>	a white eye phenotype (no italics)
<i>w⁺</i>	a red eye phenotype (no italics)
<i>w⁺</i>	a wild type <i>white</i> gene genotype (italics)
<i>w^{m4}</i>	<i>In(1)w^{m4}</i> , chromosome inversion involving <i>white</i> gene
<i>w^{m5}</i>	<i>T(1;4)w^{m5}, w^{m5}</i>
<i>y</i>	the <i>yellow</i> gene
Δ2-3	gene for the <i>P</i> element transposase source

Chapter 1: Introduction

1 Introduction

In eukaryotes, the majority of genes are present in euchromatin, packaged into irregular nucleosome arrays, and are accessible to transcriptional factors (Richards and Elgin, 2002; Kurdistan and Grunstein, 2003). Heterochromatin mostly contains repetitive DNA sequences packaged into regular nucleosome arrays and is inaccessible to transcriptional factors (Cryderman et al, 1998; Lohe and Brutlag, 1986; Sun et al, 2000; Wallrath and Elgin, 1995). The packaging state of heterochromatin is maintained after DNA replication and mitosis, and thus it is epigenetically heritable (Elgin and Grewal, 2004). This plays an important role in a variety of chromosomal functions, such as chromosome segregation and repression of large pericentric and telomeric domains, all of which help maintain genome integrity (Elgin and Grewal, 2004). Since heterochromatin is important for genome integrity, a good tool is required for the investigation of its structure and function.

In *Drosophila*, the relocation or insertion of almost any gene adjacent to domains of pericentric heterochromatin results in variable silencing of this gene, which is referred to as heterochromatic position effect variegation (hPEV) (Spofford, 1967; Weiler and Wakimoto, 1995). The *white*⁺ gene is one of the most frequently studied gene reporters of hPEV, and its phenotype is observed as patches of mutant and wild-type pigmentation in the eye (Martin-Morris et al, 1997). Second site modifiers of hPEV have been identified as candidate genes involved in the assembly of heterochromatin, therefore, hPEV provides a good tool for the investigation of heterochromatin structure and function (Martin-Morris et al, 1997). This thesis characterizes variable silencing of a P transgene in *cis* and in *trans*, and explores the relationship between gene silencing and heterochromatin.

The *P(lacW)cf^{Dplac}* enhancer trap insertion (hereafter called *Pcl*) contains a *white* mini-gene that is expressed normally in backgrounds lacking P elements (Figure 1.1) (Locke et al, submitted). However, in backgrounds containing P elements, the mini-*white* gene shows a variegated phenotype, similar to that of hPEV and is referred to as P element dependent silencing (PDS) (Locke et al, submitted). Bushey and Locke (2004) isolated two derivative alleles of *P(lacW)cf^{Dplac}* called *P(lacW)cf^{E1}* and *P(lacW)cf^{E2}*, that show a variegated phenotype even in the absence of other P elements. Both *P(lacW)cf^E* alleles show a variegated colored eye in M strains, indicating that this is *cis*-acting silencing (Figure 1.2). Moreover, both alleles produce a variegated eye when heterozygous with the parental *P(lacW)cf^{Dplac}* allele, so they also have *trans*-silencing activity (Figure 1.2) (Bushey and Locke, 2004).

Molecular analysis showed that each *P(lacW)cf^E* allele had a *gypsy* transposable element independently inserted about 1 kbp distal to *P(lacW)cf^{Dplac}* (Figure 1.1). The coincident

appearance of the *gypsy* insertions with both *cis*- and *trans*-silencing effects suggests that the *gypsy* elements are responsible for silencing effects of both $P(lacW)cf^E$ alleles (Bushey and Locke, 2004). Thus, in this study, I investigated which sequences flanking or adjacent to the insertion are required for the silencing effects of the two $P(lacW)cf^E$ alleles. To that end, I used $\Delta 2-3$ as the transposase source to mobilize the $P(lacW)$ construct of $P(lacW)cf^E$ and $P(lacW)cf^{E2}$ and generate deletions of, within, and adjacent to, this insert.

Therefore, in the introduction, I will first talk about heterochromatic position effect variegation (hPEV) and the relationship of transposable elements and heterochromatin formation. Then I will introduce $P(lacW)cf^{P^{lac}}$, $P(lacW)cf^E$ and $P(lacW)cf^{E2}$ alleles and the *cis*- and *trans*-silencing of $P(lacW)cf^E$ alleles. Lastly, I will talk about the *cis*-silencing of $P(lacW)cf^E$ alleles and the *gypsy* insertion, compare the *trans*-silencing of $P(lacW)cf^E$ alleles with other *trans*-silencing systems, and explain how $\Delta 2-3$ can be used as the transposase to generate flanking and internal deletions for this study.

1.1 Heterochromatic position effect variegation (hPEV)

Heterochromatic regions are cytologically defined by a condensed chromatin state during interphase, such as the chromocenter of polytene chromosomes, and a Barr body caused by X chromosome inactivation in human females (Pirrotta and Rastelli, 1994; Weiler and Wakimoto, 1995). Chromosomal rearrangements that place euchromatic genes next to heterochromatic environments result in variable silencing of the euchromatic genes. The cell-to-cell differences in silencing lead to a variegated phenotype. The chromosome inversion, $ln(1)w^{m4}$, hereafter referred to as w^{m4} , places the *white* (w^+) gene next to centromeric heterochromatin and this causes variable silencing and a variegated phenotype with white and colored ommatidia. The w^+ gene is necessary for red and brown pigment accumulation in individual cells, so the ommatidia are white when it is turned off. Since eye color is not necessary for viability and is easy to determine, this phenotype is advantageous to use in genetic screens.

1.1.1 SU(VAR) proteins and histone modifications are associated with heterochromatic regions

Genetic screens using w^{m4} have identified more than 150 heterochromatic suppressors ($Su(var)$) or enhancers ($E(var)$) of hPEV (Schotta et al, 2003). Mutations in $Su(var)$ s suppress the variegated phenotype and produce more colored ommatidia, whereas mutations in $E(var)$ s enhance the variegation and produce more white ommatidia. w^{m4} has also been used to identify genes whose product bound to densely stained heterochromatic regions. $Su(var)$

genes encode structural components of heterochromatin, such as the chromo domain protein HP1 (Eissenberg et al, 1990; Eissenberg and Elgin, 2000), histone methyltransferase (HMTase) SU(VAR)3-9 (Rea et al, 2000; Schotta et al, 2002), and the zinc finger protein SU(VAR)3-7 (Delattre et al, 2000). HP1, SU(VAR)3-9 and SU(VAR)3-7 are components of heterochromatin, and *Su(var)3-9* is dominant over the other two genes in a genetic hierarchy. This indicates that histone lysine methylation plays an important role in heterochromatin formation (Schotta et al, 2002).

Not only do SU(VAR) proteins localize to heterochromatic regions, but also certain variant histones and certain chemical modifications are associated with heterochromatic regions (Jenuwein and Allis, 2001; Turner 2002). Specific amino acids within nucleosomes can be acetylated, methylated, ubiquitinated and phosphorylated, and these specific modifications are associated with either heterochromatic or euchromatic regions. For instance, methylation at lysine 9 on histone H3 (K9-H3) is associated with heterochromatic regions in *Drosophila* as well as in mammals. These modifications provide a "histone code", and this "histone code" can be recognized by protein domains found within the SU(VAR)s that bind to specific types of chromatin structure (Jenuwein and Allis, 2001; Nakayama et al, 2001; Turner 2002).

In *Drosophila*, H3-K9 dimethylation was found to be enriched at heterochromatin (Schotta et al, 2002). SU(VAR)3-9 mainly controls H3-K9 dimethylation and trimethylation at pericentric heterochromatin, and the silencing potential of SU(VAR)3-9 is mainly determined by the kinetic properties of the HMTase reaction (Ebert et al, 2004). In *Su(var)3-9* null mutants, H3-K9 monomethylation is not significantly altered, whereas H3-K9 dimethylation and trimethylation are significantly reduced in the chromocenter but not in the fourth chromosome (Ebert et al, 2004). HP1 is an interaction partner of SU(VAR)3-9 and it is important for retaining SU(VAR)3-9 at pericentric heterochromatin (Schotta et al, 2002). In *Su(var)2-5* (HP1) mutants, H3-K9 mono-, di-, and trimethylation appear unaltered in the chromocenter, while H3-K9 mono- and dimethylation are dramatically increased along euchromatic arms (Ebert et al, 2004).

Together, in *Drosophila* pericentric heterochromatin is largely characterized by H3-K9 dimethylation and the chromocenter core is enriched for H3-K9 trimethylation. Also, there is evidence for *Su(var)3-9*-independent H3-K9 monomethylation at the chromocenter and additional H3-K9 methyltransferases that target the fourth chromosome (Ebert et al, 2004). Heterochromatin does not expand along the fourth chromosome, because it is modified by different methyltransferase complexes and that might be critical to this spreading effect (Ebert et al, 2004). Ebert et al demonstrate that H3-K9 dimethylation is the major mark to induce

chromatin condensation and expansion of heterochromatic structures. H3-K9 trimethylation, on the other hand, plays a minor role in heterochromatin formation but rather functions in organizing chromatin structure at centromeres.

1.1.2 SU(VAR)3-9, HP1 and SU(VAR)3-7 are associated in heterochromatin

Although heterochromatin typically silences expression of euchromatic genes, it is necessary for the appropriate expression of genes that normally reside in heterochromatin. These heterochromatic regions are typically gene poor, but they contain a few essential genes, such as *rolled (rl)* and *light (lt)* (Eberl et al, 1993; Weiler and Wakimoto, 1998). The product of *Su(var)2-5*, HP1, binds to cytologically visible heterochromatin at the centromere (Eissenberg et al, 1990; Eissenberg et al, 1992). Reducing the *Su(var)2-5* dosage suppresses heterochromatic position effect variegation and thus increases expression from a euchromatic *white⁺-lacZ* reporter transgene inserted in a heterochromatic region (Lu et al, 2000). However, reducing the *Su(var)2-5* dosage reduces gene expression from both *rolled* and *light* (Lu et al, 2000). Therefore, the heterochromatic environment is necessary for appropriate expression from genes that normally reside within it, although it is generally inhibitory to euchromatic gene expression.

The heterochromatic modifiers identified in *Drosophila* and other species have similar protein domains, such as the SET domain and chromodomain (Jacobs and Khorasanizadeh, 2002). The SET domain is a motif which was first identified as a C-terminal region of homology shared by SU(VAR)3-9, ENHANCER of ZESTE and TRITHORAX proteins. The chromodomain is a conserved chromatin repressor domain found in both HP1 and SU(VAR)3-9, and it mediates the association of HP1 and methylated lysine 9 of H3. The SET domain is found in *Su(var)3-9*, and this domain is involved in methylation at lysine 9 in H3 (K9-H3). *Su(var)3-9* has homologues in mammals (*Suv39H1* and *Suv39H2*) and in *Schizosaccharomyces pombe* (*clr4*) (Bannister et al, 2001). SU(VAR)3-9 and HP1 interact to maintain histone methylation in heterochromatic regions. This mechanism has been found in mammals, *S. pombe*, and *Drosophila*, so research in these organisms has been used to describe this common pathway. The chromodomain in HP1 and its homologue in *S. pombe*, Swi6, has affinity for H3 tails that have methylated K9. Also, HP1 has affinity for SU(VAR)3-9, so it can recruit this protein to heterochromatic regions and methylate H3 K9 (Nakayama et al, 2001). This is a feedback loop that maintains the H3 methylation. In addition, murine tissue culture results show that the three HP1 (α , β and γ) homologues act in a similar feedback loop with the two SU(VAR)3-9 homologues (SUV39H1 and SUV39H2) (Lachner et al, 2001). Although the interactions between SU(VAR)3-9, HP1 and H3 K9 methylation

suggest how heterochromatin is maintained, they do not indicate how heterochromatin formation is initiated.

Drosophila HP1 also interacts with SU(VAR)3-7, which was shown by the two hybrid assay in yeast (Delattre et al, 2000). Furthermore, HP1 and SU(VAR)3-7 were co-immunoprecipitated from nuclear extract, and SU(VAR)3-7 could be recruited by delocalized HP1 (Cleard et al, 1997; Delattre et al, 2000). Taken together, these results suggest that SU(VAR)3-9, HP1 and SU(VAR)3-7 are associated in heterochromatin (Jaquet et al, 2002). *Su(var)3-7* encodes a protein that is associated with mostly pericentric heterochromatin in embryonic nuclei and in salivary gland polytene chromosomes, and also telomeres and a few euchromatic sites (Delattre et al, 2000). The protein contains seven atypical and widely spaced zinc finger motifs in its N-terminal moiety and the BESS motif of 40 amino acids in its C-terminal moiety (Cleard and Spierer, 2001; Altschul et al, 1997). Since the BESS motif is found in proteins BEAF, SU(VAR)3-7 and Stonewall, it is named after those proteins (Jaquet et al, 2002). It has been proposed that the zinc fingers act as a DNA compacting tool to cause the repression, while the C-terminal moiety ensures heterochromatin recognition and association by dimerization through the BESS motif and interaction with other proteins (Jaquet et al, 2002). In summary, SU(VAR)3-9 can methylate lysine 9 of histone H3, which would lead to the recruitment of HP1. The binding of HP1 would in turn recruit SU(VAR)3-7, which can then compact chromatin and cause silencing (Jaquet et al, 2002).

Heterochromatin formation is necessary for genomic stability. Complete loss of HP1 in *Drosophila* is lethal; in the dying embryos chromatin bridges form between nuclei and chromosomes do not completely condense during mitosis (Kellum and Alberts, 1995). Complete loss of *Suv39h1* and *Suv39h2* in murine cells impairs chromosomal stability, viability and male fertility (Kellum and Alberts, 1995; Peters et al, 2001). Heterochromatin could prevent premature replication at the centromere that would interrupt kinetochore assembly and chromosome condensation during mitosis (Henikoff, 2000). According to this, heterochromatin is necessary for appropriate chromosome condensation and segregation during the cell cycle and thus the loss of heterochromatin leads to genomic instability. On the other hand, the change from heterochromatic to euchromatic regions may result in ectopic expression of genes that reside in heterochromatin, which in turn causes genomic instability.

1.2 Heterochromatin formation

1.2.1 Heterochromatin formation is triggered by repetitive sequences

Heterochromatin does not consist of a unique sequence that heterochromatin modifiers bind to, but repetitive sequences containing transposable elements and satellite sequences (Bartolome et al, 2002; Hoskins et al, 2002). One reason why transposable elements could cluster in heterochromatin is because heterochromatic regions have few genes and therefore transposable element insertions would be less detrimental in these regions. Insertions in gene-poor heterochromatin will rarely interrupt a gene, whereas insertions in gene rich euchromatin will interrupt a gene more frequently. Another reason why transposable elements may accumulate in heterochromatin is that ectopic recombination between transposable elements at different locations on homologous chromosomes will result in the loss or gain of genetic material, which could be harmful to the genome. Heterochromatic regions have a reduced recombination frequency, so it would inhibit ectopic recombination (Dimitri and Junakovic, 1999). In summary, heterochromatin formation could be involved in the domestication and control of transposable elements.

Heterochromatin formation is triggered by repetitive sequences (Henikoff, 2000; Dorer and Henikoff, 1994). Classic hPEV involves the translocation or insertion of euchromatic genes into tandem repeats found in centromeric and telomeric regions. Tandem *P(lacW)* repeats alone can trigger heterochromatin formation and result in silencing of the *w^{mc}* transgenes found in the repeats (Henikoff, 2000; Dorer and Henikoff, 1994). Like hPEV at other loci, reducing the *Su(var)2-5* dosage suppresses heterochromatic silencing within the *P(lacW)* repeats (Dorer and Henikoff, 1994); proximity to heterochromatic regions enhances heterochromatic silencing in the repeats (Dorer and Henikoff, 1997). Such a system also appears to act in mammals since simple TA repeats trigger heterochromatic silencing (Saveliev et al, 2003).

1.2.2 Transposable elements and heterochromatin formation

A transposable element is a DNA sequence that can replicate itself through either a DNA or RNA intermediate and this replication could lead to inverted repeats or increase gene copy number in a genome. If transposable element replication goes unchecked, it can kill the host. Either tandem repeats of a transposable element or an insertion in repetitive sequences could trigger heterochromatin formation and silencing. This relationship between repeats and heterochromatic silencing suggests that heterochromatin could have evolved as a means to control transposable elements.

Transposable elements are DNA sequences that are able to integrate into the genome at a new site within the original cell, and they include DNA transposons, autonomous retrotransposons, and nonautonomous retrotransposons (Craig et al, 2002). DNA transposons are found in bacteria and also in the genomes of many eukaryotes, including insects, worms and humans. They are generally excised from one genomic site and integrated into another by a "cut and paste" mechanism. Insertions can occur at a large number of genomic sites because sequence specificity of integration is limited to a small number of nucleotides (for example TA dinucleotides for Tc1 of *Caenorhabditis elegans*). Nevertheless, most daughter insertions occur adjacent to the parental insertion, which is called "local hopping" (Mizuuchi et al, 2002).

Active transposons encode a transposase enzyme between inverted-repeat termini. The transposase binds at or near the inverted repeats and also binds to the target DNA. It first breaks the DNA to remove the transposon from its original site, and then joins the DNA to insert the transposon into a new site. The phosphodiester bonds between the transposon and flanking DNA are hydrolyzed to liberate 3'-OH residues, which then carry out the attack at the new site (Mizuuchi et al, 2002). The two strands at the new site are attacked at staggered sites, therefore, the inserted transposon is flanked by small gaps. When the gaps are filled in by host enzymes, short duplications of sequence are created at the target site. The length for target site duplications (TSDs) is often characteristic for a particular transposon (Craig et al, 2002).

To move a piece of DNA, the reactions use two main classes of recombinase enzymes. The first class is called conservative. The enzymes do not require high-energy cofactors, the total number of phosphodiester bonds is unchanged, and no DNA degradation or resynthesis occurs. The integrase protein of bacteriophage λ , Cre recombinase, and Flp recombinase fall into this class. The second class is the transposases that catalyze a set of reactions necessary for DNA transposition, including the transposases of Mu, P elements, and the Tc1/mariner family, etc. These elements generally transpose to genomic sites less than 100 kbp from their original sites (the *Drosophila* P element), but some are able to make distant transposition (the fish Tc1/mariner element) (Mizuuchi et al, 2002; Curcio and Derbyshire, 2003).

Many genomes contain a large number of active elements, but they remain reasonably stable. In large genomes with active elements, only a small fraction of the genome consists of protein-coding and regulatory sequences, such as mice and humans. In small genomes, genes constitute a large fraction but transposable-element mobility is low, with exceptions like *Drosophila* (International Human Genome Sequencing Consortium, 2001; Mouse Genome

Sequencing Consortium, 2002; SanMiguel et al, 1996). However, the host places further controls on mobility in both of these scenarios. At least two control mechanisms are known. One is cosuppression, suppression of both the expression of an introduced transgene and its endogenous homologs (Jiang, 2002; Garfinkel et al, 2003); the other is methylation of mobile elements (Bestor, 2003).

1.2.3 RNAi is involved in heterochromatin formation

How tandem repeats are recognized by heterochromatin proteins is not yet understood. Small RNAs associated with the RNA interference (RNAi) pathway are involved in heterochromatin formation in some organisms. RNAi results from the formation of small double stranded RNAs (siRNAs) and it was originally observed in *C. elegans* (Hannon, 2002). An RNase III enzyme, Dicer, mediates the processing of longer double stranded RNA precursors into siRNAs. The siRNAs are then incorporated into the multi-protein RNA-induced silencing complex (RISC). The unwinding of the double stranded RNA by a helicase activity in the complex targets RISC to specific mRNAs.

Studies in *Schizosaccharomyces pombe* showed that the silencing of heterochromatin in pericentromeric regions and in the mating type locus requires components of the RNAi machinery, Argonaute, Dicer and RNA-dependent RNA polymerase (Rdp1) (Volpe et al, 2003). These components were also shown to be necessary for the maintenance of silencing of a marker gene (*Ura6*) located adjacent to heterochromatin repeats, lysine 9 methylation and association of the HP1 homolog Swi6 with the marker gene. Furthermore, expression of an artificial hairpin-forming inverted repeat sequence homologous to a *Ura6* gene was sufficient by itself to direct silencing of the *Ura6* gene, which was accompanied by H3 lysine 9 methylation and Swi6 binding (Schramke and Allshire, 2003).

Although it's known that RNAi plays a role in heterochromatin formation in *S. pombe*, the mechanism for that is not yet known. What sheds some light on this mechanism is the purification of an RNA-protein complex that contains RNAi components and appears to be involved in heterochromatin formation in *S. pombe* (Verdel et al, 2004). The complex contains Argonaute, Chp1 and a newly identified protein, Tas3. It was already known that Chp1 is required for methylation of histone H3 K9 and localization of Swi6 and it also binds to centromeric repeats. Like mutations in RNAi components, mutations in *Chp1* and *Tas3* genes interfered with silencing of a centromeric *Ura4* gene.

RNAi plays a role in heterochromatin formation in *S. pombe* and plants, which makes it likely that similar mechanisms operate in other organisms as well. Pal-Bhadra et al (2004) have shown that the RNAi machinery is required for heterochromatic silencing and

localization of histone H3 lysine 9 methylation and HP1 to pericentromeric heterochromatin in *Drosophila*. Mutations in components of the RNAi machinery (*aubergine*, *piwi* and *homeless*) result in loss of silencing. The level of methylated histone H3 lysine 9 in the nucleus is reduced and HP1 is redistributed along the entire polytene chromosome. Thus far, there is only indirect evidence for involvement of RNAi in heterochromatin formation in mammals. Treatment of mammalian cells with ribonuclease resulted in loss of HP1 binding and changes in the pattern of histone H3 methylation (Maison et al, 2002).

It's known that tandem repeats are frequently associated with heterochromatin formation. A model relates RNAi mediated chromatin silencing to this observation (Martienssen, 2004). The model suggests that RdRP uses downstream primers sequentially to generate new substrates for the RNAi machinery and this leads to exhaustion of siRNAs from single-copy sequences. For tandem arrays, however, the downstream repeats provide a continuous supply of full length substrate, therefore, RNAi can occur and thus mediate heterochromatin formation.

1.3 P element dependent phenotypes

About 45% of the human genome and 10% of the *Drosophila* genome is composed of interspersed transposable element sequences (Lander et al, 2001; Labrador and Corces, 1997). For many years, P transposable elements of *Drosophila* have been a powerful tool for insertional mutagenesis and a method to link phenotype with genomic sequence (Spradling et al, 1999). In *Drosophila*, the P element is one of the best-characterized transposable elements; it encodes proteins that can interact with the host's proteins to catalyze and regulate transposition. Recombinant DNA techniques may be used to remove DNA sequence within the P element and replace them with sequences from other genes, and researchers have been able to transform *Drosophila* with P element constructs carrying genes of interest (Spradling et al, 1999).

1.3.1 P promoter specific repression of *P-lacZ*

The *white*⁺ gene has been studied in classical chromosomal contexts, and P element-mediated germ line transformation of the *white*⁺ gene has also been widely used for studying the *white*⁺ gene (Pirrota and Rastelli, 1994). P constructs that have the *lacZ* gene fused to the P promoter (*P-lacZ*) have been used to measure expression from the P elements (Lemaitre and Coen, 1991; Lemaitre et al, 1993). Originally, these constructs acted as enhancer traps to detect nearby transcriptional enhancers that activate the P promoter (Lemaitre and Coen, 1991; Lemaitre et al, 1993). The P promoter activity results in *lacZ*

expression and β -galactosidase activity, and therefore, the expression pattern for the *P-lacZ* insert will mimic the expression pattern for the endogenous gene that the enhancer usually acts on (Lemaitre and Coen, 1991; Lemaitre et al, 1993). However, when crossed to P strains, the expression from the *P-lacZ* reporter is repressed (Lemaitre and Coen, 1991; Lemaitre et al, 1993). Thus, this repression caused by P elements in the genetic background is P promoter specific.

1.3.2 P element dependent silencing of *P(lacW)ci^{P_{lac}}*

It has recently been shown that the *P(lacW)ci^{P_{lac}}* enhancer trap insertion, which was a P construct with a *lacZ* reporter gene and a *white* mini-gene marker inserted about 3 kbp distal to the *ci* transcription unit on chromosome 4 (Figure 1.1), is expressed normally in backgrounds lacking P elements (Locke et al, submitted). However, in backgrounds containing P elements, the mini-*white* gene shows a variegated phenotype, similar to that of hPEV (Locke et al, submitted). This phenomenon is referred to as P element dependent silencing (PDS) (Locke et al, submitted).

A different *white*⁺ transgene insert, *P(hsp26-pt-T)ci^{2-M1021.R}*, is inserted in this same region where the *P(lacW)ci^{P_{lac}}* construct is inserted and is located 140 bp closer to *ci* compared to *P(lacW)ci^{P_{lac}}*. This *white*⁺ transgene also shows variable silencing in the presence of P elements. In this transgene, the *white* transcript is driven by a heat shock promoter, so a *white* gene promoter-specific silencing mechanism cannot explain P element dependent silencing (PDS), and the PDS is distinct from repressing the expression of *P-lacZ* constructs, which is P promoter specific. Instead, a change in chromatin structure to this region that causes the *white*⁺ gene silencing is proposed (Locke et al, submitted). The PDS of single *white* transgene insertions is limited to the region distal to *ci* locus on chromosome 4, and it does not represent a general phenomenon that occurs at other P inserts (Locke et al, submitted). This suggests there is a "local" effect of the adjacent sequences in this region required for silencing the inserted transgenes (Locke et al, submitted).

In summary, P elements cause variegated expression of the *w*⁺ transgene in *P(lacW)ci^{P_{lac}}* and *P(hsp26-pt-T)ci^{2-M1021.R}*, which are both inserted about 3 kbp distal to the *ci* gene (Locke et al, Submitted). In M type flies, which don't contain P elements, expression from either *w*⁺ transgene produces a uniformly red eye phenotype. In P type flies, which have P elements, expression from both *w*⁺ transgenes produces a variegated eye phenotype because *w*⁺ is randomly silenced in some ommatidia (white) but expressed in others (red). This variegated silencing resembles heterochromatic position effect variegation (hPEV) where changes in local chromatin structure can variably silence gene expression. Thus, a

change in chromatin structure to this region that causes the *white*^{*} gene silencing is proposed (Locke et al, submitted).

Changes in chromatin structure can affect gene expression regardless of its sequence. *P(lacW)cf^{Dplac}* has the *mini-white*^{*} gene that uses portions of the original *w*^{*} promoter, whereas *P(hsp26-pt-T)cf^{2-M1021.R}* has the promoter from *hsp70*^{*} fused to the *w*^{*} transgene. Since different P constructs with different promoters are silenced, a specific DNA promoter sequence is not required to induce silencing. Moreover, chromatin structure within a region depends on its chromosomal location. *Mini-white*^{*} expression from *P(lacW)* inserted at other locations is not silenced by the presence of P elements (Locke et al, Submitted). Changing the chromosomal location changes the chromatin structure and PDS doesn't occur. Thus, P elements could cause *w*^{*} silencing within the P inserts in this region due to the unique chromatin structure in this region.

hPEV can be caused by chromosomal rearrangements or insertions that place genes next to telomeres and centromeres where heterochromatin normally forms. The *ci* gene is located next to the centromere on chromosome 4, so transgene inserts at this locus could behave similarly to hPEV caused by heterochromatin formation at centromeres. Bushey recovered mutations in *Su(var)2-5* and *Su(var)3-7* that suppress PDS at *P(lacW)cf^{Dplac}* (Bushey and Locke, 2004). These mutations occur in known heterochromatic modifiers, confirming that changes in chromatin structure cause the silencing.

1.4 *P(lacW)cf^{Dplac}*, *P(lacW)cf^{E1}* and *P(lacW)cf^{E2}* alleles

Bushey and Locke (2004) isolated two derivative alleles of *P(lacW)cf^{Dplac}* called *P(lacW)cf^{E1}* and *P(lacW)cf^{E2}*, which show a variegated phenotype even in the absence of other P elements (Figure 1.2). To compare the variegation of *P(lacW)cf^E* alleles with hPEV, Bushey and Locke (2004) tested whether classic modifiers of hPEV would affect the silencing of *P(lacW)cf^E* alleles. An extra Y-chromosome, *Su(var)205* alleles or *Su(var)3-7* alleles, all of which suppress hPEV, also suppressed the variegated eye phenotype of both *P(lacW)cf^E* alleles. However, *Su(var)3-9* alleles, which strongly suppress hPEV, didn't affect the variegated phenotype of *P(lacW)cf^E* alleles (Bushey and Locke, 2004). Therefore, the variegated expression of the *white* gene in *P(lacW)cf^E* alleles has a mechanism that is similar to, but distinct from, typical hPEV (Bushey and Locke, 2004). Further investigation is needed to determine the molecular similarities and differences for this silencing and that of hPEV.

1.4.1 The *cis*-silencing and *trans*-silencing of $P(lacW)cf^1$ and $P(lacW)cf^2$ alleles and the *gypsy* insertion

Both $P(lacW)cf$ alleles show a variegated colored eye in M strains, indicating this is *cis*-acting silencing. When a wild type chromosome 4 is heterozygous with $P(lacW)cf^{Dplac}$, the *white* gene of $P(lacW)cf^{Dplac}$ is expressed as demonstrated by the red eye phenotype. However, when $P(lacW)cf^1$ or $P(lacW)cf^2$ is heterozygous with the parental $P(lacW)cf^{Dplac}$ allele, the *white* gene of $P(lacW)cf^{Dplac}$ is variably silenced as shown by the variegated eye phenotype in Figure 1.2 (Bushey and Locke, 2004). Since this silencing is due to sequences on the homolog, instead of on the same chromosome, it is called *trans*-silencing. Therefore, both $P(lacW)cf^1$ and $P(lacW)cf^2$ alleles have a *trans*-silencing effect.

It is important to note, however, that when $P(lacW)cf^1$ or $P(lacW)cf^2$ is heterozygous with the parental $P(lacW)cf^{Dplac}$ allele, the *cis*-silencing of $P(lacW)cf^1$ or $P(lacW)cf^2$ still exists, and in addition to that, $P(lacW)cf^1$ or $P(lacW)cf^2$ can *trans*-silence $P(lacW)cf^{Dplac}$. Thus, in order to investigate the *trans*-silencing only, I needed to avoid the phenotype of the *cis*-silencing first. To that end, I generated $w^- P(lacW)cf^1$ and $P(lacW)cf^2$ derivatives that were caused by a partial or complete deletion of the *white* gene of $P(lacW)cf^1$ and $P(lacW)cf^2$. Since the *white* gene of $P(lacW)cf^1$ and $P(lacW)cf^2$ derivatives was deleted, their *cis*-silencing had no effect on the eye color phenotype, and thus I was able to investigate their *trans*-silencing ability only.

Molecular analysis showed that each $P(lacW)cf$ allele had a *gypsy* transposable element independently inserted about 1 kbp distal to $P(lacW)cf^{Dplac}$ (Figure 1.1). The coincident appearance of the *gypsy* insertions with both the *cis*- and *trans*-silencing effect suggests that the *gypsy* elements are responsible for the silencing effects of either $P(lacW)cf$ allele (Bushey and Locke, 2004).

1.4.2 The insulator function of *gypsy* element

The *gypsy* retro-transposon of *Drosophila* is responsible for mutations that affect genes located throughout the genome (Modolell et al, 1983). The *yellow* gene is one of them; it is useful for studying gene regulation because it is not essential for viability. Expression of the *yellow* gene during *Drosophila* development is controlled by a series of enhancers. Enhancers that control *yellow* expression in the wings, body cuticle, and larval pigmented structures are located in the 5' region of the gene, while enhancers that control *yellow* expression in the bristles and tarsal claws are located in the intron (Geyer and Corces, 1992). When *gypsy* is inserted in the 5' region of *yellow*, the distal wing and body cuticle enhancers fail to activate transcription but the larval enhancer proximal to the promoter is not affected.

Studies have identified a specific region within *gypsy* that is responsible for this effect, and this region is necessary and sufficient for the effect to occur (Geyer et al, 1988; Smith and Corces, 1995). This effect is not specific to *yellow*. When these sequences are present on both sides of a *white* transgene, they can buffer the expression of this transgene from chromosomal position effects (Roseman et al, 1993). These data suggest that *gypsy* might act as an insulator element and cause mutations by separating enhancers and promoters into different chromatic domains (Gdula et al, 1996).

The *gypsy*-induced mutant phenotypes can be reversed by mutations in *suppressor of Hairy-wing (su(Hw))* gene, indicating that this protein is required for mediating *gypsy* effects on transcription (Lewis, 1981). The *su(Hw)* protein is a nuclear protein present in all *Drosophila* cells; it contains 12 zinc fingers in the central region and two acidic domains located in the amino- and carboxyl- terminal regions of the protein (Harrison et al, 1993). The insulator region of *gypsy* contains an octamer motif flanked by AT tracts (Geyer et al, 1988; Peifer and Bender, 1988). The *su(Hw)* product interacts directly with the octamer motif and the flanking AT tracts help to make contacts of *su(Hw)* with sequences on both sides of the octamer (Spana et al, 1988; Mazo et al, 1989; Dorsett, 1990; Spana and Corces, 1990). Since *su(Hw)* is a nuclear protein and can bind to DNA, its localization might correspond to endogenous insulators that are important for organizing the genome into higher order chromatin domains (Gdula et al, 1996).

Other second-site suppressors or enhancers should help elucidate how the *gypsy* insulator acts. One mutation is *modifier of mdg4 (mod(mdg4))*; it affects phenotypes caused by *gypsy* insertion at different loci including *yellow* (Georgiev and Gerasimova, 1989). Mutations in *mod(mdg4)* affect enhancers located both distal and proximal to the promoter, instead of only distal enhancers. This effect is specific for *gypsy*-induced mutations and requires the presence of the *gypsy* element. A copy of the *gypsy* element lacking the insulator sequences doesn't have this effect, and the *gypsy* insulator is sufficient to elicit this effect (Gerasimova et al, 1995). Moreover, *mod(mdg4)* mutations fail to enhance the *yellow* mutant phenotype when null mutations of the *su(Hw)* gene are present. These data suggest that the *mod(mdg4)* protein might bind to the *gypsy* insulator and interact directly with the *su(Hw)* protein (Gdula et al, 1996).

When *su(Hw)* and *mod(mdg4)* are both present, the *gypsy* insulator affects enhancer function in one direction. With respect to the promoter, only enhancers distal from the insulator are repressed. In the absence of *mod(mdg4)*, enhancers located both distal and proximal to the insulator are inactivated. Thus, *su(Hw)* alone, when bound to the insulator, can affect enhancers in both directions; *mod(mdg4)* appears to change this effect from acting

in both directions to acting in one direction. To summarize, *su(Hw)* and *mod(mdg4)* can interact with each other and this interaction may be an integral part of the *gypsy* insulator.

1.4.3 The *trans*-silencing system at the *ci*, *yellow* and *brown* loci

Trans-acting silencing systems need to be introduced here because the mutations produced in this study are *trans*-silencing. There are three major systems in *Drosophila* and each is described here.

The *ci* gene is necessary for appropriate anterior: posterior boundaries forming in the embryo and imaginal discs. In each compartment *ci* is expressed only in the anterior compartment. Amorphic mutations knocking out *ci* function are recessive lethal. However, gain-of-function mutations where *ci* is ectopically expressed in the posterior compartment of wing disks produce a recessive phenotype that affects the adult wing veins. This *ci* wing vein phenotype is recessive because a wild type *ci*⁺ allele in *trans* can repress expression from alleles such as *ci*¹ or *ci*^{F79} (Locke and Tartof, 1994). These recessive *ci* alleles ectopically express *ci* in the posterior compartment when homozygous, because no repression occurs in *trans*. The ectopic *ci* expression causes an interruption in the L4 wing vein. These recessive *ci* alleles are also ectopically expressed when rearrangements, such as *ln(1;4)w^{ms}*, prevent pairing between chromosome 4 homologues. Thus, the repression in *trans* requires pairing between chromosome 4 homologues. This phenomenon is termed transvection and it occurs at other loci such as *yellow* and *brown* (Henikoff, 1997; Henikoff and Comai, 1998).

Transvection at the *yellow* (*y*) locus was first demonstrated for the *y*² allele (Lindsley and Grell, 1968). In the *y*² allele, a *gypsy* element is inserted at -700 bp from the transcription start site, which separates wing and body enhancers from the promoter. *y*² flies have yellow cuticle in the wing and body due to the insulator effect of *gypsy*. Geyer et al (1990) characterized a number of *yellow* mutant alleles that can complement the *y*² wing and body phenotypes. They found that all complementing alleles have the wing and body enhancers although the *y* promoter was deleted or damaged (Geyer et al, 1990). *y*^{80d} is one of those, and it is a *y*² derivative that deletes part of *gypsy*, the *y* promoter, and all of the transcribed region, but it leaves the wing and body enhancers intact. This allele can complement *y*², suggesting that it is the wing and body enhancers that act in *trans* and drive transcription of the *y*² promoter in the wing and body cuticle (Geyer et al, 1990). The *y*^{80b} allele is also derived from *y*² and deletes part of *gypsy*, the *y* promoter, and 5' part of the transcribed region. It can also complement *y*², but does not complement *y*² transgenes inserted at ectopic locations. This indicates that complementation of *y*^{80b} and *y*² appears to be pairing dependent (Geyer et al, 1990).

The eye color gene *brown* (*bw*) is another example. When *bw* is translocated next to heterochromatin (*bw^v* rearrangements), the rearrangements can variably silence the *bw^v* allele on the rearranged homolog and also the *bw⁺* on the normal homolog (Henikoff and Dreesen, 1989) so that the *bw^v* acts as a dominant allele. Since *bw⁺* transgenes located at ectopic locations are not suppressed by the rearrangements, the *trans*-silencing of *bw⁺* in *bw^v* rearrangements is also pairing-dependent (Dreesen et al, 1991). When the *bw⁺* gene contained in a *bw^v* rearrangement was excised by P-element mobilization, silencing of the *bw⁺* gene in *trans* was still observed. This suggested that *trans*-silencing does not require the presence of the *bw⁺* gene itself in *cis* to the rearrangement (Dreesen et al, 1991). These results indicate that *bw⁺* is sensitive to repression by heterochromatin, and PEV can occur in *cis* as well as in *trans* (Duncan, 2002).

1.4.4 $\Delta 2-3$ transposase can be used to study sequences required for the *cis*- and *trans*-silencing of both *P(lacW)c^F* alleles

We do not yet know which sequences within or adjacent to the *P(lacW)c^F* construct of *P(lacW)c^F* alleles are required for the *cis*-silencing effect, and which are required for the *trans*-silencing effect. It should be possible to select for adjacent deletion of the *gypsy* element by mobilizing the *P(lacW)c^F* construct using the $\Delta 2-3$ transposase source. A variety of such adjacent deletion alleles could then be examined as to how those deleted sequences affect the *cis*- and *trans*-silencing of *P(lacW)c^F* on the homolog.

P elements produce a transcript in the germ line that is composed of four exons, labeled 0, 1, 2 and 3. This transcript encodes a transposase that catalyzes the excision of *P* elements, as DNA intermediates, and inserts them back into the genome. Replication occurs when the gap left by the excised *P* element is repaired using the sister chromatid as a template. In the soma, an alternative transcript is produced, which retains the intron between exons 2 and 3. The somatic transcript encodes a truncated polypeptide that lacks transposase activity, so transposition does not occur in the soma.

$\Delta 2-3$ is a *P* element that had the intron between exons 2 and 3 removed. When transformed back into the *Drosophila* germline it produces a transcript that encodes transposase in somatic cells. In the soma, transposase activity causes *P* element transposition, such that a *P* element insert containing a *w⁺* transgene results in a mosaic eye colour phenotype. Thus, expressing $\Delta 2-3$ in somatic tissues can result in transposition of *P* elements and this produces a mosaic phenotype. In this study, however, I am interested in using $\Delta 2-3$ in the germline to produce excision mutations.

1.5 Overview of this study

The $P(lacW)cf^{P_{lac}}$ enhancer trap insertion, which was a P construct with a $lacZ$ reporter gene and a *white* mini-gene marker inserted about 3 kbp distal to the *ci* transcription unit on chromosome 4 (Figure 1.1), is expressed normally in backgrounds lacking P elements (Locke et al, submitted). However, in backgrounds containing P elements, the mini-*white* gene shows a variegated phenotype, similar to that of hPEV (Locke et al, submitted). This phenomenon is referred to as P element dependent silencing (PDS) (Locke et al, submitted). The PDS of single *white* transgene insertions is limited to the region distal to *ci* locus on chromosome 4, and it does not represent a general phenomenon that occurs at other P inserts (Locke et al, submitted). This suggests there is a "local" effect of the adjacent sequences in this region required for silencing the inserted transgenes (Locke et al, submitted).

Bushey and Locke (2004) isolated two derivative alleles of $P(lacW)cf^{P_{lac}}$ called $P(lacW)cf^{E1}$ and $P(lacW)cf^{E2}$, which show a variegated phenotype even in the absence of other P elements. To compare the variegation of $P(lacW)cf^E$ alleles with hPEV, Bushey and Locke (2004) tested whether classic modifiers of hPEV would affect the silencing of $P(lacW)cf^E$ alleles. An extra Y-chromosome, *Su(var)205* alleles or *Su(var)3-7* alleles, all of which suppress hPEV, also suppressed the variegated eye phenotype of both $P(lacW)cf^E$ alleles. Both $P(lacW)cf^E$ alleles show a variegated colored eye in M strains, indicating this is *cis*-acting silencing (Figure 1.2). Moreover, both alleles produce a variegated eye when heterozygous with the parental $P(lacW)cf^{P_{lac}}$ allele, so they also have *trans*-silencing activity (Figure 1.2) (Bushey and Locke, 2004). Molecular analysis showed that each $P(lacW)cf^E$ allele had a *gypsy* transposable element independently inserted about 1 kbp distal to $P(lacW)cf^{P_{lac}}$ (Figure 1.1). The coincident appearance of the *gypsy* insertions with both the *cis*- and *trans*-silencing effect suggests that the *gypsy* elements are responsible for the silencing effects of both $P(lacW)cf^E$ alleles (Bushey and Locke, 2004).

If the *gypsy* element is responsible for the *cis*-silencing of the $P(lacW)cf^E$ alleles, then its deletion from the $P(lacW)cf^E$ alleles should revert the variegated eye phenotype to a uniformly red eye phenotype (w^+). The construct $\Delta 2-3$ can be used as the transposase source to mobilize the $P(lacW)$ construct in the $P(lacW)cf^{E1}$ and $P(lacW)cf^{E2}$ alleles. Imprecise excisions cause deletions within or flanking the $P(lacW)$ construct. The flanking deletions may include the *gypsy* element. I used $\Delta 2-3$ as the transposase source to mobilize the $P(lacW)$ construct in the $P(lacW)cf^{E1}$ and $P(lacW)cf^{E2}$ alleles and recovered mutants that showed a red-eye mutant phenotype, rather than the typical variegated eye color.

Both $P(lacW)_{cf}$ alleles can silence the $P(lacW)_{cf^{plac}}$ allele in *trans* (Bushey and Locke, 2004). I presume sequences within or adjacent to the $P(lacW)$ construct of $P(lacW)_{cf}$ alleles are required for this *trans*-silencing to occur. As with the first screen, I used $\Delta 2-3$ as the transposase source to mobilize the $P(lacW)$ construct of $P(lacW)_{cf^{E1}}$ and $P(lacW)_{cf^{E2}}$ and generate deletions of, within, and adjacent to, this insert. These deletions will produce white-eye flies (w^-), so from this screen I recovered w^- mutants. One w^- derivative, $E1R4$, has lost the whole *gypsy* element and the distal end (~ 0.5 kbp) of the $P(lacW)$ transposon. Southern blot hybridization results suggest that there is no second site insertion of the $P(lacW)$ construct on chromosome 4. Therefore, the red eye phenotype coincides with the loss of the *gypsy* element, suggesting that the *gypsy* element is responsible for the *cis*-silencing of the $P(lacW)_{cf}$ alleles.

The structures of w^- derivatives showed that the excision of the whole $P(lacW)$ construct of $P(lacW)_{cf}$ alleles still permits *trans*-silencing, and that the degree of *trans*-silencing appears to correlate with the amount of the sequence in the $P(lacW)$ construct shared by the mutant allele and the $P(lacW)_{cf^{plac}}$ allele.

Pedigree of mutant alleles used/created in this study

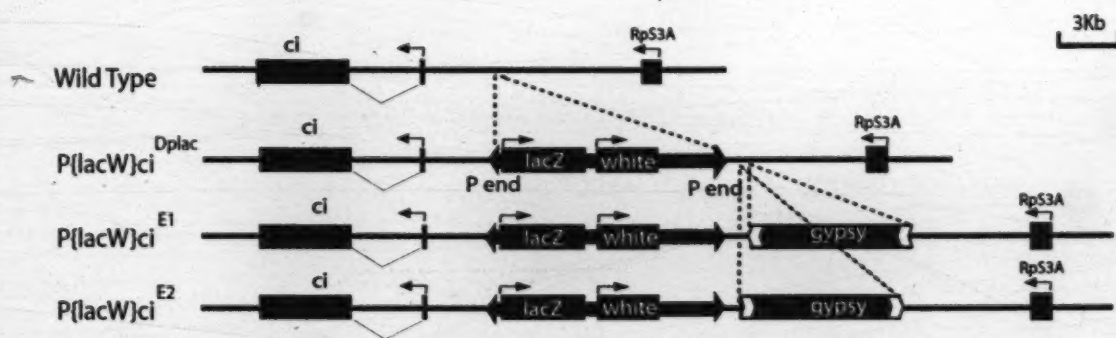
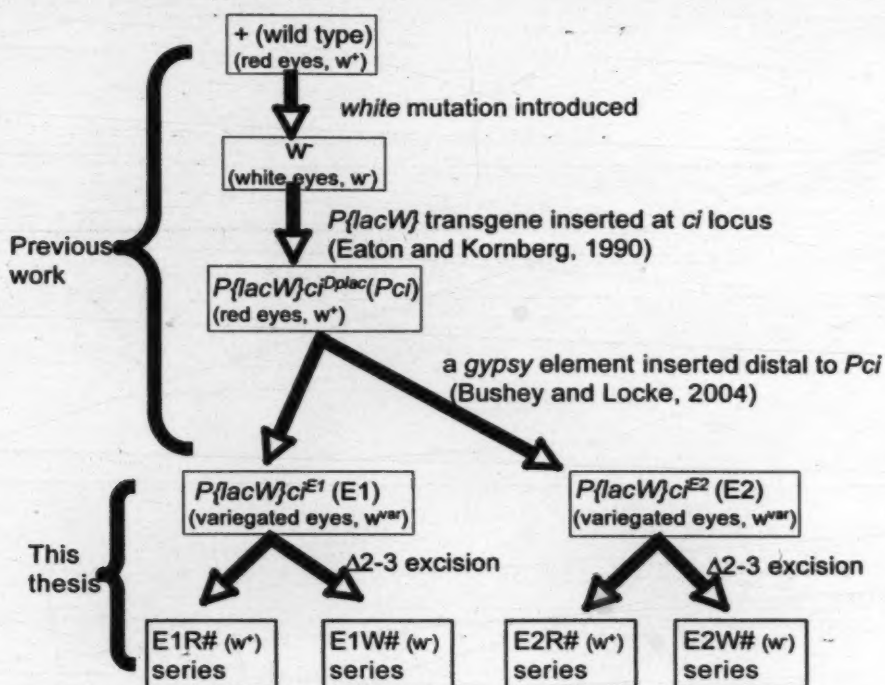
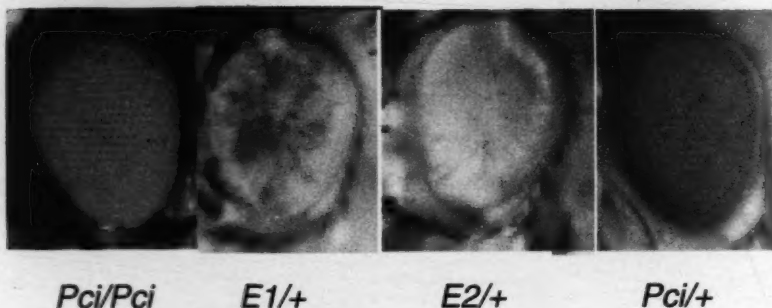


Figure 1.1 Pedigree of mutant alleles used/created in this study and the structures of four alleles (wild type, *Pci*, *E1* and *E2*). Diagrams representing wild type chromosome 4, the *Pci* chromosome, the *E1* chromosome and the *E2* chromosome. The chromosome is depicted as a thick line; genes are depicted as solid blocks. Horizontal arrows indicate the promoters of genes; open block arrows on the *gypsy* sequence indicate orientation of the LTRs.

A. *Cis*-silencing



B. *Trans*-silencing

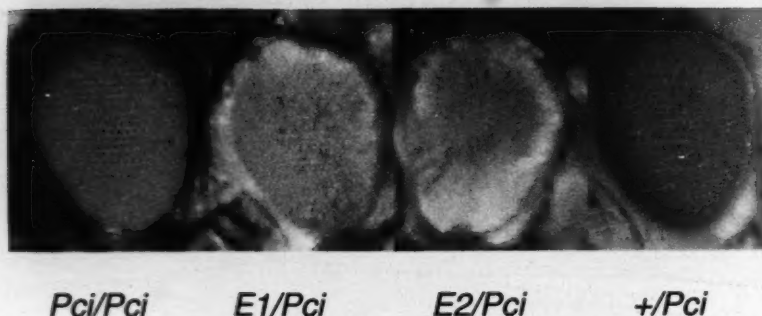


Figure 1.2 Pictures of *Drosophila* eyes show the *cis*- and *trans*-silencing of *P(lacW)cf* alleles. All stocks used in this study is in a white background. The *P(lacW)cf^{Δlac}*, *P(lacW)cf^{E1}* and *P(lacW)cf^{E2}* alleles are referred to *Pci*, *E1* and *E2*, respectively. The genotype for the fly shown in each picture is indicated beneath each picture.

A. *E1* and *E2* alleles show *cis*-silencing, when heterozygous with a wild type chromosome 4 (+). *Pci/+* flies have a solid red eye phenotype, whereas *E1/+* and *E2/+* flies have a variegated eye phenotype.

B. *E1* and *E2* alleles can *trans*-silence the *Pci* allele. Since *Pci/+* flies generate a solid red eye phenotype and *E1/+* and *E2/+* flies generate a variegated eye phenotype, *E1/Pci* and *E2/Pci* could have shown the additive eye phenotype of the *E* allele and the *Pci* allele, a solid red eye phenotype. Instead, both *E1/Pci* and *E2/Pci* show a variegated eye phenotype, which suggests the ability of *E1* and *E2* alleles to *trans*-silence the *Pci* allele. All the pictures are provided by Daniel Bushey and are used with his permission.

Chapter 2: Materials and Methods

2 Materials and Methods

2.1 Fly maintenance and stocks

Drosophila cultures and crosses were maintained at 21°C on standard yeast/sucrose medium (each litre contains 10 g agar, 100 g sucrose, 100 g brewers yeast, 100 ml of 1 g/l chloramphenicol, 10 ml propionic acid, 4.3 g NaH₂PO₄ and, 2.7 g Na₂HPO₄).

The three transgenes used in this study are *P(lacW)cf^{Dplac}*, *P(lacW)cf^{E1}* and *P(lacW)cf^{E2}*. The structures of *P(lacW)cf^{Dplac}*, a chromosome 4 *P(lacW)* insertion, and the two derivative alleles, *P(lacW)cf^{E1}* and *P(lacW)cf^{E2}* alleles are shown in Figure 1.1 (Bushey and Locke, 2004). The *P(lacW)cf^{Dplac}* allele is a *P* construct containing *lacZ^{PT.W}*, *w^{mc}*, *amp^R* and *ori*; the construct is inserted between an 8 base pair duplication starting at 79894 (GTCTGTAC) in AE03854.3 which is about 3 kbp upstream from the *ci* locus on chromosome 4 (Locke et al, Submitted). *P(lacW)cf^{E1}* and *P(lacW)cf^{E2}* both have a *gypsy* element inserted about 1 kbp distal to the *P(lacW)* construct (Bushey and Locke, 2004). In *P(lacW)cf^{E1}*, the *gypsy* element is inserted between a four base pair duplication (TACA) starting at 81294 (AE003854), in *P(lacW)cf^{E2}* the *gypsy* element is inserted between a four base pair duplication (TATA) starting at 80747 (AE003854) (Bushey and Locke, 2004). *P(lacW)cf^{Dplac}* and *P(lacW)cf^{E1}* are homozygous viable, while *P(lacW)cf^{E2}* is homozygous lethal and is maintained over *Dp(2;4)ey^D, ey^DAlp^{ey^D}*. Other genetic markers are described in FlyBase. All the stocks used in this study are in a *white* background (with a white eye phenotype) unless stated otherwise.

The genotypes of the stocks used in this study are found in Appendix1 at the end of this chapter.

2.2 Screen of Δ2-3 mobilized *P(lacW)cf^{Dplac}*

The *P(lacW)cf^{Dplac}* transposon was mobilized by crossing *w; Sb e Δ2-3 / TM3 Ser; ci¹ey^R* males to *w; P(lacW)cf^{E1}* (or *w; P(lacW)cf^{E2} / Dp(2;4)ey^D, ey^DAlp^{ey^D}*) females (detailed crossing scheme in Figure 2.1). F1 males containing both the Δ2-3 transposase source and the *P(lacW)cf^{Dplac}* construct were crossed to *w; dp; e; ci¹ey^R* virgin females. In the F2 generation, *w; dp/+; e/+; ci¹ey^R / P(lacW)cf^{Dplac}* males normally had variegated eyes. Rare mutant red-eye or white-eye progeny were collected for further investigation.

2.3 Eye pigment assay

I determined the level of *white* gene expression by measuring the amount of brown eye pigment. The measurement was made based on the method of Locke et al (1988). For each

genotype, we collected 5-9 day old flies, stored them at -70°C, and recovered their heads. I assigned three replicate samples containing 20 heads each. The pigment in each sample was extracted for 36-48 hours of continuous agitation in 500ml of acidified methyl alcohol (AMA: 1% v/v HCl in methanol). Then 2.5 µl of 1% H₂O₂ was added to each sample, which was then incubated at room temperature for 90-120 minutes. After incubation, the absorbance at 470 was measured for each sample in a LKB Ultraspec II spectrophotometer.

2.4 Single fly extraction protocol

I added 50 µl of "squishing buffer" (10mM TRIS-HCl pH 8; 1mM EDTA pH 8, 25mM NaCl + freshly added Proteinase K at 200 µg/ml) to a 0.6 ml PCR tube containing a single fly. I mashed the fly with a pipette tip by adding only a small amount of the 50 µl volume in the tip and squishing the fly with the tip still attached to the pipetman. When the fly seems to be well mashed (separate pieces, some color usually seen in the buffer), the remaining volume in the tip was added. For embryo preps, I used the same protocol, in a volume of 10 µl.

Then I incubated the tubes at 37°C for 45 minutes and at 99°C for 10 minutes. I placed the tube in the thermocycler (Eppendorf AG 22331 Hamburg) and ran the program "FLY1" (Lid at 100°C, 1. 37°C for 45 minutes, 2. Link to Fly 2). This program automatically links to "FLY2" (Lid at 100°C, 1. 99°C for 10 minutes, 2. 4°C).

The preparation was now ready for use as a PCR template. A reaction volume of 1.0 µl is usually sufficient template for a single PCR reaction of 30 µl to 50 µl total volume (Di Franco C et al).

2.5 DNA extraction from flies using a QIAGEN™ kit

Typically, 25-50 flies were ground in a 1.5 ml microfuge tube using 180 µl of ATL (tissue lysis buffer) and a blue disposable pestle. Then 30 µl of proteinase K solution was added and the tube was vortexed before incubation at 60°C for 60 minutes. During the incubation, the tube was vortexed occasionally.

After incubation, 200µl of buffer AL (lysis buffer) was added, the tube was mixed by vortexing and incubated at 70°C for 15 minutes. Then 200 µl of room temperature 95% ethanol was added and the contents were mixed by vortexing. The entire mixture was pipetted into a spin column. The column was centrifuged at 8000 rpm for 1 minute, and the flow-through was discarded. 500 µl of AW1 (wash buffer 1) was added and the column was centrifuged at 8000 rpm for 1 minute. After discarding the flow-through, 500 µl of AW2 (wash buffer 2) was added and the column was centrifuged at 14000rpm for 3 minutes. The flow-

through was discarded and a new catch microfuge tube was placed underneath the column. 200 μ l of AE (elution buffer) was added to the column and allowed to equilibrate for 1 minute. This elution step was repeated with another 200 μ l AE (elution buffer). A 1/10 dilution from the 400 μ l of genomic DNA provided enough template for a typical PCR amplification (Vogelstein, B. et al, 1962)

2.6 Polymerase chain reaction (PCR)

Fragments of genomic DNA were amplified by polymerase chain reactions in 30 μ l reactions (1 μ l DNA template and 29 μ l PCR reaction mixture) containing about 0.1 μ g of genomic DNA with 0.75 units Taq polymerase at an annealing temperature of 5°C-10°C below the melting temperature of the primers, with 45 sec denaturation at 95°C and 2 min synthesis at 72°C for <2 kbp fragments or longer times at 68° for >2 kbp DNA fragments. Amplified DNA products were size separated on agarose gels (modified from Molecular Cloning by Sambrook, Fritsch and Maniatis).

The sequence for each primer used to characterize the mutant alleles is listed in Table 2.1.

2.7 Sequencing of PCR products

To determine the sequence of a PCR product, first I size separated the fragment by gel electrophoresis and excised the DNA band containing the desired fragment from an agarose gel. Then I placed the gel fragment into a clean microfuge tube. The tube was weighed before and after the gel slice to determine the volume of the agarose gel slice. The DNA was extracted from the gel slice using the Qiagen gel extraction kit. I followed the directions supplied with the kit.

The reaction mix is set up in a 0.6 ml tube as shown in Appendix 3. The reaction is run in the thermocycler using program SEQ (1. 95°C 30 second, 2. 50°C 15 second, 3. 60°C 60 second, 4. Go to 1, Rep 29, end).

After the cycling was complete, I added 2 μ l of EDTA/Na-acetate + 80 μ l of 95% ethanol to the tube. The tube was vortexed briefly to mix the solution and placed in the freezer for about an hour. After precipitation, the tube was centrifuged for 15 minutes and the ethanol was decanted. The pellet was usually not visible. The pellet was washed with 200 μ l of 80% ethanol and vortexed briefly. The tube was centrifuged again for 15 minutes and then the supernatant was discarded. After the ethanol was carefully removed using an empty tip, the tube was left to dry on the bench top (modified from Molecular Cloning by Sambrook, Fritsch and Maniatis).

2.8 Large-scale genomic DNA extraction protocol

Typically, 50-100 flies were ground in a 1.5 ml tube with a blue disposable pestle in 500 μ l of homogenization buffer (200 mM EDTA pH8, 100mM TRIS pH7.5, 1% SDS), preheated to 60°C. I transferred contents to a 2 ml screw cap tube, rinsed homogenizer with an additional 100 μ l of buffer, and added to the tube contents. The 2 ml tube was incubated at 60 °C for 30 minutes.

After the incubation, I immediately added 600 μ l of CHCl_3 (all work with organic solvents was in the fume hood, and while wearing gloves). I mixed the contents for 1 minute by inverting the tube. I centrifuged the tube for 3 minutes (using a Jouan microfuge, model number A-14) and removed CHCl_3 layer with a fine tip transfer pipette. I avoided taking the fly bits as this would clog the transfer pipette. CHCl_3 and phenol waste were discarded into a waste container for proper disposal. Then I added 600 μ l of phenol, and mixed the contents by inverting the tube. I gently broke up the fly layer with a tip if the layer didn't break apart during inversion. I centrifuged the tube for 3 minutes, and removed phenol layer with a transfer pipette. I repeated phenol extraction step. Then I added 600 μ l of CHCl_3 , mixed the contents by inversion, and centrifuged the tube for 3 minutes. I carefully transferred only the aqueous layer to a 1.5 ml tube using a wide-bore transfer pipette, being careful to leave all fly bits in the old tube.

Then I added enough TE to bring the volume to 500 μ l. After adding 50 μ l of 3M NaCl and 1000 μ l of cold 95% ethanol, I mixed the contents by inversion, thoroughly but gently, until the nucleic acid precipitated out of solution. I spun the tube for 1 minute to collect at the bottom of the tube. I decanted the ethanol and let the pellet dry for 10 - 30 minutes, but not more, or the pellet would be difficult to redissolve.

By inversion, I resuspended the pellet in 200 μ l TE by inversion. DNA was left overnight to dissolve completely. RNA was degraded by adding 20 μ l of RNaseA (0.5 mg/ml) and incubated the tube at 37 °C for 15 minutes. After incubation, I added 200 μ l of phenol/ CHCl_3 and mixed the contents by inversion. I centrifuged the tube for 3 minutes and transferred the aqueous layer (~ 200 μ l) to new tube using a wide-bore tip.

Then I added 20 μ l of 3M NaCl and 450 μ l of 95% ethanol. I mixed the contents by inversion until the DNA had precipitated. After centrifuging the tube for 1 minute, I decanted ethanol and washed DNA briefly with 80% ethanol and let it dry. Then I resuspended the DNA in 50 μ l of TE and allowed the pellet to dissolve completely. I ran a 5 μ l sample on a gel to test quality and quantity of DNA. The DNA was then ready for digestion with restriction

enzymes (modified from Molecular Cloning by Sambrook, Fritsch and Maniatis, Ko, David, and Akashi).

2.9 Southern transfer

The restriction enzyme digested genomic DNA was separated on a 0.8% agarose gel in 1X TAE pH 8.3 (0.04M Tris-acetate, 0.001M EDTA) for 17 hours at 35V. The gel was photographed with a ruler alongside, using software GeneSnap from Syngene. The image was saved as a jpeg file and also printed out for a record.

The gel was soaked with gentle shaking in 0.25M HCl for 10 minutes, then rinsed in H₂O. The DNA was denatured by soaking, with gentle shaking, in 0.5M NaOH / 1.5M NaCl for 30 minutes. Then the gel was rinsed in H₂O, and gently shaken in 1.5M NaCl / 0.5M TRIS-HCl pH 7.5 for 30 minutes.

During the previous steps, I cut the Genescreen Plus membrane to exactly the same size as the gel and equilibrated the membrane in 10X SSC for 10 minutes. Then I placed 3 long strips of Whatman filter paper (Electrophoresis and blotting paper, AHLSTROM paper group, Grade 238) (the same width as the gel) onto a glass plate that rested on a plastic container. I wetted the paper with 10X SSC, and partially filled the container with 10X SSC. I placed the gel, with wells facing down, on top of the paper. Then I used a glass pipette to gently roll out any air bubbles that may be present between the gel and the paper. I used strips of parafilm along the sides of the gel to prevent any transfer of liquid to the paper above except through the gel. I placed the pre-wetted membrane on top of the gel. Again, I removed any air bubbles by rolling over the gel and membrane with a pipette. I placed 3 pieces of pre-wetted Whatman paper (the same size as the gel) on top of the membrane, and removed air bubbles. I stacked paper towels (cut to the appropriate size) on top of the paper, and placed a glass plate and a weight on the top. I made sure that the buffer reservoir contained enough liquid. I left the DNA to transfer overnight.

Next day, I removed the paper, and carefully marked the lanes on the membrane with a pencil and labeled the membrane for identification purposes. I rinsed the membrane in 0.5M NaOH for 1 minute, then rinsed in 0.2M TRIS-HCl pH 7.5. I allowed the membrane to dry completely in the 60°C incubator on a piece of filter paper. It was then ready for hybridization (modified from Molecular Cloning by Sambrook, Fritsch and Maniatis).

2.10 Random primer labeling and Southern hybridization

2.10.1 Random primer labeling

I placed DNA and dH₂O in a labeled screw-cap tube and incubated the tube in the boiling water for 3-5 minutes, and then placed it immediately on ice. I added 5X OLB (details in Appendix 5), ³²P-dCTP, and Klenow as shown in Appendix 4. I flicked the tube to mix the contents, centrifuged the tube, and incubated it at 37°C for ~3 hours (modified from Molecular Cloning by Sambrook, Fritsch and Maniatis).

2.10.2 Pre-hybridization

I placed the membrane in a hybridization bottle or a covered dish with enough hybridization solution to cover the membrane. I used bottles to do the hybridization so I used nylon mesh to prevent overlapping membranes from trapping probe. I pre-hybridized the membrane at 65°C for 30 minutes for DNA blots (modified from Molecular Cloning by Sambrook, Fritsch and Maniatis).

2.10.3 Hybridization

I boiled the labeled probe in the kettle for 3-5 minutes. Then I diluted the probe in ~500 µl of hybridization solution, and added the entire amount immediately to the container or tube. I was careful not to add the probe directly onto the membrane, as this may cause a region of high background. I allowed hybridization reaction to proceed overnight at 65°C in regular hybridization solution in a rotating motion (modified from Molecular Cloning by Sambrook, Fritsch and Maniatis).

2.10.4 Stringency washes

I placed the membrane into a covered dish containing pre-warmed Wash I (details in Appendix 5). I incubated it with shaking, at 65°C for 30 minutes. The radioactive hybridization solution was poured carefully into the waste bottle. The membrane was transferred into a new dish containing pre-warmed Wash II (details in Appendix 5). It was incubated with shaking, at 65°C for ~30 minutes. The radioactive wash buffer I was poured carefully into the waste bottle.

Then I transferred membrane into a new dish containing pre-warmed Wash III (details in Appendix 5). It was incubated, with shaking, at 65°C for ~30 minutes. I monitored the levels of radioactivity with the Geiger monitor. The wash buffer was diluted and poured down the drain. If a lower level stringency wash was desired, I only used Wash 1, monitoring with the

Geiger to check the radioactivity level. I then placed the wet membrane onto a piece of wet filter paper, and wrapped it well with Saran™ wrap, so that the membrane cannot dry out. I checked with the Geiger monitor to see if any signal can be detected. If the signal was strong, a lower exposure time (~4 hours) should be sufficient. I placed the wrapped membrane into a film cassette with orientation dots (modified from Molecular Cloning by Sambrook, Fritsch and Maniatis).

2.10.5 Autoradiography

I placed X-ray film (Fuji medical X-ray film, REF 03G010) on top of the wrapped membrane, clipped the upper left corner of the film for orientation, also used fluorescent dots as orientation dots, and placed the cassette at -80°C. I allowed the cassette to thaw before attempting to open it and develop the film using Kodak X-OMAT 2000 processor (modified from Molecular Cloning by Sambrook, Fritsch and Maniatis).

2.11 Removal of probe from membrane

The Southern blot was denatured in excess Strip I (0.4M NaOH) at 42°C for 30 minutes. Then the filter was neutralized in excess Strip II (0.2M Tris pH 7.8, 0.1×SSC, 0.1% SDS) at 42°C for 30 minutes. The strip washes were poured down the sink. Special care was taken to drain off all excess NaOH, because NaOH would reduce RNA probes to NTPs (modified from Molecular Cloning by Sambrook, Fritsch and Maniatis).

2.12 Genetic crosses testing for the presence of translocations

Translocation tests were done for all five w^+ mutants.

For w^+ *E1* derivatives, w ; *E1R/E1R* males were crossed to w ; *dp; e; ci¹ey^R* females to generate w ; *dp/+; e/+; E1R/ci¹ey^R* flies. The w ; *dp/+; e/+; E1R/ci¹ey^R* males were selected and crossed to w ; *dp; e; ci¹ey^R* females to generate progeny for examination. The same crosses were done to all four w^+ *E1* derivatives. For *E2R1*, w ; *E2R1/M^{67g}* males were crossed to w ; *dp; e; ci¹ey^R* females to generate w ; *dp/+; e/+; E2R1/ci¹ey^R* and w ; *dp/+; e/+; M^{67g}/ci¹ey^R* flies, which had red eyes and white eyes, respectively. The w ; *dp/+; e/+; E2R1/ci¹ey^R* males were selected and crossed to w ; *dp; e; ci¹ey^R* females to generate progeny for examination.

If a translocation was present and involved chromosome 3, then the *E1R* or *E2R* chromosome (red eye phenotype) would segregate with the e^+ chromosome. Likewise, if the translocation involved chromosome 2, the *E1R* or *E2R* progeny would only show *dp*⁺ phenotype. If the *E1R* or *E2R* progeny were unlinked to *e* and *dp* then they don't contain translocations involving chromosome 2 or 3.

The progeny were first divided into two groups by eye color. $E1R/ci^1ey^R$ or $E2R/ci^1ey^R$ flies had red eyes, whereas ci^1ey^R/ci^1ey^R flies had white eyes. Then within each group, the progeny were divided into four subgroups by dp and e phenotypes, which were dp^+e^+ , dp^+e^- , dp^-e^+ , dp^-e^- , respectively.

2.13 Examination of Polytene chromosomes

Fly crosses were set up in vials to generate larvae of the desired genotypes. Parents were transferred every day to ensure that larvae grow under uncrowded conditions. Third instar larvae used for polytene chromosome squashes were those that crawled out of medium and up on the wall of the vial and showed relatively less movement.

Selected larvae were first washed in distilled water and then dissected in 45% acetic acid. One pair of forceps was used to grip the mouth-hooks of the larvae, while the other pair was used to grip the middle-point of the body. The mouth-hooks were pulled out of the body in a quick motion. The salivary glands should follow the mouth-hooks with white fat body tissue and maybe other tissues as well. The attached tissue was removed to the point where little, or no fat body tissue remained attached to the salivary glands.

A drop of dye (Lactio Acetic Orcein Stain) was added to the center of a glass slide, and the glands were transferred into the dye. A cover slip was put on the dye gently, to avoid any air bubble trapped between the cover slip and the glass slide. Using the wooden end of a dissecting needle, a zigzag line was drawn on the cover slip from up left corner to bottom right corner to help spreading of chromosomes. After this, thumb pressure was applied on top of the cover slip for 10 seconds to squash the chromosomes and help them adhere to the slide. Then the chromosomes were ready for examination under phase contrast microscope.

The quality of the polytene chromosomes in the spreads was checked with a Zeiss Axiophot photomicroscope (West Germany, EL-Einsatz, 451888) with the 40X dry objective using phase contrast illumination (phase 2). Selected chromosomes were photographed with a 40X or 100X oil objective (phase 3) and images were recorded with a Nikon Coolpix 995 digital camera (3.2 Megapixel) and were saved as jpeg files.

The translocation, $T(1;4)w^{ms},w^{ms}$, (referred to w^{ms} hereafter) was selected as a positive control for a chromosome 4 translocation. It has an easily seen and identified chromosome 4 translocation. Male w^{ms} were crossed to $+/+$ Oregon-R females and the female larvae (heterozygous for the translocation) were examined for polytene chromosomes squash and were used as a control for translocation. Oregon-R $+/+$ larvae were used as a control for normal polytene chromosomes.

Three slides were made for each genotype. Sets of pictures were taken for at least five nuclei on each slide, and for each nucleus, the set of pictures includes an overview of the nucleus and a close-up for chromosome 4 (where the mutation is located). Thus, for each genotype, there are at least 15 sets of pictures, but only one close-up was chosen to present in Figure 3.3 and Figure 3.13.

2.13.1 Polytene chromosome squashes for *E1W10* and *E1W17*

E1W10 and *E1W17* are both recessive lethal, so they are both stocked over *Dp(2;4)ey^D, ey^DAlp^{ey^D}* (called *ey^D* hereafter). *E1W10/ey^D* males and *E1W17/ey^D* males were crossed to *+/+* (Oregon-R stock) females to generate *E1W10/+* and *+/ey^D* larvae, and *E1W17/+* and *+/ey^D* larvae, respectively. *ey^D* squashes were identified by their characteristic aberration, which I identified by examining the larvae from the following cross. *Dl(4)M101-62F/ey^D* (*Dl(4)M101-62F* is referred to *M^{62F}* hereafter) males were crossed to *+/+* females to generate *M^{62F}/+* and *+/ey^D* larvae. Since *+/ey^D* larvae hatch much earlier than *M^{62F}/+* larvae, the larvae that hatched earlier were collected and used for squashes. Three slides for *+/ey^D* were made and used as an *ey^D* control.

Table 2.1: Primers used to characterize mutant alleles recovered from screen of mobilized *P(lacW)c^{Dlac}*

Primers	5' Sequence 3'
JS3	TACTCAGTTCAAATCTTG TG
JS1	ATACATATGTTTCATTACGG
Pout	ATGATGAAATAACATAAGG
B1B	CGGTGTAGGTCGTTGCTCC
F1C	GGATAATACCGCGCCACATA
F1D	AAGGGAATAAGGGCGACACG
14201F	GTTGACGATGTGCAGCTAA
14383R	GTATGATAACCGGCGGACCC
14638F	ACTGGCAGCTCTGAGGTGAA
15124F	TGAGCACCAGACAGCCAGGAT
15581F	TGACGGTCTTGCCCTTCTGC
16217F	CGGTATGTGTCGCTCGTTGC
16255R	CAGAATAGTCCGCGTGTCG
16793R	CTCAGCCGCAGACAATTG
16803F	TCGCAGTTCCTATAGATCAAGT
17261R	TGAGTGAGAGGTAATCGAAAG
F2C	ACTCCCTGACAATAAAAACGAA
F2	TCAGAAAGCAGACCAAACAG
F2B	GAGAACAGAGAAATAGCGGC
18558R	CATCAATCCGGTAGGTTTCC
B2	TCAGACGATTCAATTGGCACC
F3D	GTCACGCAACTCGCCGCAC
20993F	CTCTTCGCTATTACGCCAG
F3C	ATGTGCTGCAAGGCGATTAA
THE3	GTACTCCCACTGGTATAGCC
JS5	ACATTCATAAAGGGTCTGGA
JS2	CAATATACATACAAACGC
G68R	TATGAACAATGCTGACGCGC
JLO11	GAAGCCCTATACTCAGTGGT
JLO13	CAAACATATGGAGGTAACAG
X	GATGCTTCAATCTGCAGC
13148F	AGGGGTTCCGCGCACATTTC
11668R	TCTCAGTTCGGTGTAGGTCC
12720R	TCTTACTGTCATGCCATCCG
13415R	TACACAAGGAACAAACACTGG
17314F	TAAGTTCAATGATGTCCAGTGC
18700F	AGGCGTTAGGGTCAATGCGG
19740F	CCCAGCGACCAGATGATCAC

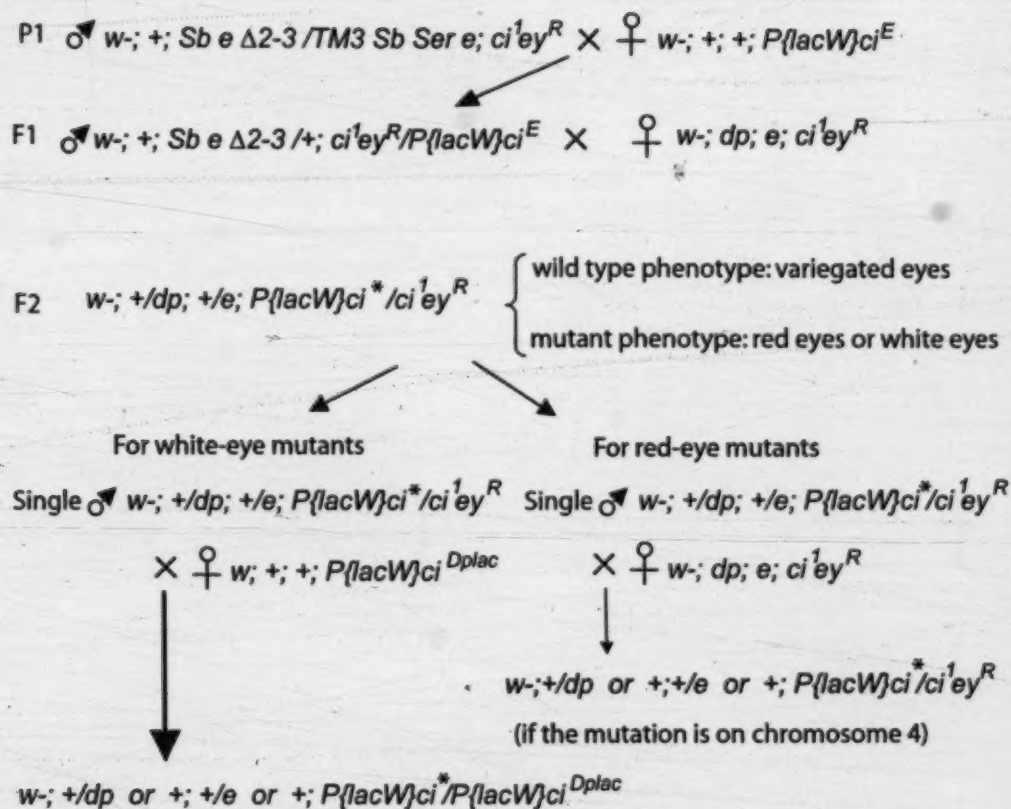


Figure 2.1 Schematic describing the screen for creating w^+ and w^- derivatives from $E1$ and $E2$ alleles. See Materials and Methods for details. In the P1 generation, males carrying $\Delta 2-3$ were crossed to females carrying $P\{lacW\}ci^E$. In the F1 generation, males containing both $\Delta 2-3$ and $P\{lacW\}ci^E$ were crossed to $w^-; dp/dp; e/e; ci^1 ey^R / ci^1 ey^R$ females. In the F2 generation, males with no $\Delta 2-3$ but with $P\{lacW\}ci^E$ were scored and the mutants (white-eye or red-eye) were recovered from the rest (variegated-eye). Mutations recovered were kept for molecular examination if they were determined to be on chromosome 4. Mutations with a white-eye phenotype were tested for their ability to *trans*-silence.

Chapter 3: Results

3 Results

The $P(lacW)c^{E1}$ ($E1$) and $P(lacW)c^{E2}$ ($E2$), which have acquired a *gypsy* transposon and the appearance of a variegated phenotype, show a *cis*-silencing in these two alleles. Both $E1$ and $E2$ alleles can also silence the *Pci* allele in *trans* (Bushey and Locke, 2004). I presume sequences within or adjacent to $P(lacW)$ of $E1$ and $E2$ alleles are required for *cis*- and/or *trans*-silencing to occur. Therefore, I used $\Delta 2-3$ as the transposase source to mobilize $P(lacW)$ of $E1$ and $E2$ and generate deletions of, within, and adjacent to, $P(lacW)$. These were identified as either w^+ or w^- phenotype progeny. From the w^+ derivatives, I could test whether the excision of the *gypsy* element would result in the loss of the *cis*-silencing on the nearby $P(lacW)$. For the w^- $E1$ and $E2$ derivatives, I then correlated their *trans*-silencing ability with their mutant structure in order to determine which sequences within or adjacent to $P(lacW)$ were required for the *trans*-silencing.

3.1 The *cis*-silencing of the $P(lacW)c^E$ alleles

3.1.1 Eye pigment and the beginning mutants and stocks

Unless stated otherwise, all the fly stocks used in this study have a loss of function *white* mutation that would normally lead to an unpigmented white eye (w^-) phenotype. In a w^- background, the mini-*white* (w^+) gene of $P(lacW)c^{P^{lac}}$ (Pci) allele is expressed generating a uniformly red eye phenotype (w^+). However, the mini-*white* (w^+) gene in the $P(lacW)c^{E1}$ ($E1$) and $P(lacW)c^{E2}$ ($E2$) alleles is variably silenced producing a variegated eye phenotype. The level of *white* gene expression is measured by the amount of pigment in the eye. When there is no *Pci* transgene in the background, flies are white-eyed (w^-) and have an eye pigment value that is about zero (Figure 3.1).

One copy and two copies of *Pci* produce increasing levels of pigmentation in the eye, therefore, pigment level can be used to determine whether the fly has one copy or two copies of *Pci* (Figure 3.1). Both $E1/+$ and $E2/+$ flies show a variegated phenotype while that of $Pci/+$ flies don't. This demonstrates that both $E1$ and $E2$ alleles have a *cis*-silencing effect (Figure 1.2).

The $E1/Pci$ heterozygote shows a variegated phenotype whereas the $+/Pci$ flies don't. This suggests that the $E1$ allele is partially *trans*-silencing w^+ expression from the *Pci* homolog. The result is similar for $E2/Pci$ flies. This indicates that both $E1$ and $E2$ alleles also have the ability to *trans*-silence the w^+ mini-gene at the same location on the homolog (Figure

1.2). The *trans*-silencing will be dealt with in the next section, while the *cis*-silencing is the topic of this section.

3.1.2 Gypsy and the *E1* and *E2* alleles

The *E1* and *E2* alleles were derived from the *Pci* allele and consequently have retained *P(lacW)* (Figure 1.1). The *E1* and *E2* alleles have acquired a *gypsy* transposable element inserted about 1 kbp distal to the *P(lacW)* insert (Bushey and Locke, 2004). The insertion of this *gypsy* element was co-incident with the appearance of the variegated phenotype, thereby strongly suggesting the *gypsy* is the cause of the *cis*-silencing in these two alleles. I could confirm this causal connection if I could excise the *gypsy* element and correlate it with the loss of the *cis*-silencing on the nearby *P(lacW)*, which would result in a white⁺ phenotype. Excision of *gypsy* could be accomplished using $\Delta 2-3$ to cause a deletion adjacent to the *P(lacW)* insertion.

3.1.3 A genetic screen for w⁺ derivatives of *E1* and *E2* alleles (loss of *cis*-silencing)

If the *gypsy* element is responsible for the *cis*-silencing of the *E1* and *E2* alleles, then its deletion from the *E1* and *E2* alleles should revert the variegated eye phenotype back to a uniformly red eye phenotype (w⁺). A deletion of the *gypsy* element could be accomplished by an imprecise excision of the *P(lacW)* transposon. Such imprecise excisions are well known in *Drosophila* and can be easily generated. The transposase source $\Delta 2-3$ can be used to mobilize the *P(lacW)* insert in the *E1* and *E2* alleles. Imprecise excisions could result in deletions within or flanking the *P(lacW)* insert. The flanking deletions may include the *gypsy* element. If the *gypsy* element is responsible for the *cis*-silencing effect of the *E1* and *E2* alleles, then its deletion should generate a red eye phenotype.

I used $\Delta 2-3$ as the transposase source to mobilize the *P(lacW)* insert in the *E1* and *E2* alleles and recovered mutants that showed a red-eye mutant phenotype, rather than the typical variegated eye color of *E1* and *E2* alleles. The *P(lacW)* transposon was mobilized from *Pci* by crossing *w*; *Sb e Δ2-3 / TM3, Ser*; *ci¹ ey^R* males to *w*; *E1* (or *w*; *E2/ ey^P*)⁺ females (detailed crossing scheme in Figure 2.1). F1 males containing both the $\Delta 2-3$ transposase source and the *E1* and *E2* insertion were crossed to *w*; *dp*; *e*; *ci¹ ey^R* virgin females. In the F2 generation, *w*; *dp/+*; *e/+*; *ci¹ ey^R* / *E1* males normally had variegated eyes. Rare mutant red-eye progeny were collected and investigated further.

⁺ *ey^P* is the short form of *Dp(2;4)ey^P, ey^P Alp^{no}*

In the progeny of this cross a red-eye phenotype can be caused by several different types of events, including the transposition of *P(lacW)* to other chromosomes. I eliminated most of these unwanted events by selecting against lines where the red-eye phenotype failed to segregate with chromosome 4. I identified those mutants where the phenotype was linked to chromosome 4 by crossing *w; dp/+; e/+; ci¹ey^R/P(lacW)ci^Δmutated* males showing a red-eye phenotype with *w; dp; e; ci¹ey^R* virgin females. If all the red-eye flies showed no *ci¹ey^R* phenotype, we concluded that the *white^{*}* mutation was on chromosome 4. Then the *ci¹ey^R/P(lacW)ci^Δmutated* males were crossed to *w; ey^D/Dl(4)M101-62F* females to generate *w; P(lacW)ci^Δmutated/ey^D* flies. Then *w; P(lacW)ci^Δmutated/ey^D* males were crossed to *w; P(lacW)ci^Δmutated/ey^D* females to produce a stock of *w; P(lacW)ci^Δmutated/ey^D*. For those red-eye mutants that did segregate with chromosome 4, the mutations could be caused by deletions within or adjacent to the *Pci* insertion or the transposition of *P(lacW)* to a different location on chromosome 4.

In this screen, I scored about 9,700 potential excision flies and recovered 82 red-eye flies (*w^{*}* mutants). A summary of the results is shown in Table 3.1. From these 82 mutants, only five had the mutant phenotype segregating with chromosome 4. Four were derived from *E1*, termed *P(lacW)ci^ΔE1R1*, *P(lacW)ci^ΔE1R2*, *P(lacW)ci^ΔE1R3* and *P(lacW)ci^ΔE1R4*; one was derived from *E2* termed *P(lacW)ci^ΔE2R1*. They are hereafter referred to as *E1R1*, *E1R2*, *E1R3*, *E1R4* and *E2R1* in this study (R=red).

3.1.4 Characterizing the five *w^{*}* mutant alleles by PCR, Southern blots, genetic crosses to detect translocations and polytene chromosome squashes

Each of the five *w^{*}* mutants was characterized by PCR, Southern blots, genetic crosses to detect translocations and polytene chromosome squashes.

I used amplifiable PCR primer pairs and DNA sequencing to define the extent of deleted regions at or near the *Pci* insertion. First, I used PCR to determine which sequences were deleted in the region where the *gypsy* element and *P(lacW)* are located. This included PCR primer pairs present i) on both ends of the *gypsy* element, ii) in the region between the *gypsy* element and the *P(lacW)* insert, and iii) within the *P(lacW)* insert itself.

If both primer sequences were present, then a PCR product of the predicted size should be obtained. If there was a deletion between the primers, then the amplified product would be smaller in size. If either primer complementary sequence was deleted then no amplification product would be produced. Once I knew where the deleted region was, I used PCR primers just outside the deleted region to amplify across the deletion breakpoints and sequence the resulting fragment. Comparing the sequence of the fragment spanning the deletion with that

of the undeleted region, I could determine the exact size of the deletion. The extent of these deletions for the five w^+ derivatives will be presented.

Southern blots were used to identify the size of genomic restriction fragments present in each mutation and to determine whether there is a second site insertion of the $P(lacW)$ transgene. In the $P(lacW)$ construct, the *amp* gene is in the distal end and the *lacZ* gene is in the proximal end. I used these two gene sequences as template DNAs to make two probes in order to detect the $P(lacW)$ insert(s) in the genome. Therefore, if there is only one copy of $P(lacW)$ in the genome, there will be two bands showing up on the Southern blot. In contrast, if there is a second site insertion of $P(lacW)$, there will be more than two bands on the blot.

Genetic crosses to detect translocations were done for all five w^+ mutants. For w^+ *E1* derivatives, $w; E1R/E1R$ males were crossed to $w; dp; e; ci^1 ey^R$ females to generate $w; dp/+; e/+; E1R/ci^1 ey^R$ males, which were then crossed to $w; dp; e; ci^1 ey^R$ females to generate progeny for examination. The same crosses were done to all four w^+ *E1* derivatives and to *E2R1* ($w; E2R1/M^{679}$) as well.

If a translocation was present and involved chromosome 3, then the *E1R* or *E2R* chromosome (red eye phenotype) would segregate with the e^+ chromosome. Likewise, if the translocation involved chromosome 2, the *E1R* or *E2R* progeny would only show dp^+ phenotype. If the *E1R* or *E2R* progeny were unlinked to *e* and *dp* then they don't contain translocations involving chromosome 2 or 3. The results for all five w^+ mutants are shown in Table 3.2 (more details in Materials and Methods).

The polytene chromosomes of all five w^+ mutants were also characterized. *E1R1*, *E1R2*, *E1R3* and *E1R4* are all stocked as homozygotes; *E2R1* is a recessive lethal and is stocked over M^{679} . Females from four of the five stocks were crossed to Oregon-R $+/+$ males to generate larvae of the desired genotypes: *E1R1/+*, *E1R2/+*, *E1R3/+*, and *E1R4/+*. Larvae of those genotypes were chosen to prepare polytene chromosome squash slides. M^{679} chromosome appears indistinguishable from the normal chromosome 4 control in polytene chromosome squash, so *E2R1/M^{679}* polytene chromosomes should have the same phenotype as *E2R1/+* polytene chromosomes. Since *E2R1* is stocked over M^{679} , *E2R1/M^{679}* larvae, instead of *E2R1/+* larvae, were used to prepare polytene chromosome squash slides. Wild type Oregon-R $+/+$ larvae were used as a control for normal polytene chromosomes (more details in Materials and Methods).

The five w^+ mutations will be described individually.

E1R4 - PCR results showed that for the primer pair located in the distal end of *P(lacW)*, no amplified product was observed, suggesting that there was a deletion within *P(lacW)* that involved its distal end. For the primer pair located outside the deleted region, the PCR product was 0.5 kbp smaller than the expected size, indicating the deletion was 0.5 kbp in size. For primer pairs present on both ends of the *gypsy* insertion, no amplified products were seen, suggesting the *gypsy* insertion was deleted in *E1R4*. I used the PCR primers just outside the deleted region to amplify across the deletion breakpoints and sequence the resulting fragment. Comparing the sequence of the fragment spanning the deletion with that of the undeleted region, the sequence of the fragment spanned from genomic sequences distal to the *gypsy* element to those proximal to the *gypsy* element and to the sequences within *P(lacW)*. These results indicated that derivative *E1R4* has lost the whole *gypsy* element and the distal end (~0.5 kbp) of the *P(lacW)* transposon (Figure 3.2).

Since PCR showed *E1R4* has lost the whole *gypsy* element, *E1R4* is comparable to *Pci* control. Moreover, *E1R4* has a 0.5 kbp deletion in the distal end of the *Pci* insertion, so the size of the band derived from the distal end of the *Pci* insertion in *E1R4* is 0.5 kbp smaller than the corresponding band in *Pci*. Southern blot results showed the two bands of expected sizes for *AvaI*, *MscI* and *SnaBI* digestions (Figure 3.2). For *BclI* digestion, one band is of the expected size (5.9 kbp) while the other one (17.3 kbp) is larger than the expected (10.1 kbp). The 10.1 kbp band is derived from the distal end of *P(lacW)*. The appearance of the 17.3 kbp band suggested that the *BclI* site adjacent to the distal end of *P(lacW)* was not involved in the *BclI* digestion (labeled with an X), whereas the next available one was (Figure 3.2).

In the genetic crosses involving *E1R4*, testing for linkage due to translocations, all four subgroups (*dp⁺ e⁺*, *dp e⁺*, *dp⁺ e*, *dp e*) in both sexes were observed (Table 3.2), demonstrating that this mutation was not a translocation involving chromosome 1, 2, or 3.

My examination of the polytene chromosomes of *E1R4/+*, showed no chromosome aberrations when compared with Oregon-R *+/+* normal control chromosomes. There was no extra chromosome 4 material on other chromosome arms, and there was no distortion or aberration on chromosome 4 either. The mutant chromosome 4 appeared indistinguishable from the normal chromosome 4 control (Figure 3.3).

Therefore, *E1R4* has lost the *gypsy* element adjacent to the *Pci* insertion, and since this loss coincided with the reversion from a variegated-eye back to a red-eye, I presume these sequences are required for the *cis*-acting variegated phenotype of *E1* allele. Although I didn't recover a *gypsy* deletion mutation for *E2*, I assume a similar mechanism causes *cis*-acting silencing in *E2* allele, as well.

E1R3 - For *E1R3*, PCR characterization showed that for primer pairs located on both ends of the *gypsy* element and within *P(lacW)*, amplified products were of expected sizes, suggesting that the *gypsy* element was still present, and there was no deletion within the *Pci* insertion. However, for primer pairs present on both ends of *P(lacW)*, no amplified products were observed, indicating that breakpoints were located in the two ends of *P(lacW)*. Furthermore, Southern blot results showed two new bands, neither of which were parental derived from the *Pci* insertion (Figure 3.4). The size of the two band suggested that the *P(lacW)* insert could simply be inverted at the original insertion site.

I examined this possibility by PCR. I used primer pairs JS5-11668 and 20993-JS1, because they are amplifiable for the inverted *P(lacW)* but not for the original *Pci*. PCR amplification showed a 1.0 kbp band in *E1R3* lane and no band in *E1* and H_2O lanes for primer pair JS5-11668, and a 0.7 kbp band in *E1R3* lane and no band in *E1* and H_2O lanes for primer pair 20993-JS1 (Figure 3.5). These results confirmed that the *E1R3* mutation was an inversion of *P(lacW)*.

Then I extracted DNA from the 1.0 kbp and 0.7 kbp bands and sequenced them. Comparing the sequencing results with the *E1* sequence, I found the proximal genomic sequence and the 31 bp repeat of *P(lacW)* were intact but they were connected to the distal end (*Amp* end) of *P(lacW)*, and in the meantime, the distal genomic sequence and the 31 bp repeat of *P(lacW)* were intact but they were connected to the proximal end (*lacZ* end) of *P(lacW)*. My conclusion from these results was that the *P(lacW)* insert was indeed inverted at the original insertion site. Therefore, the *E1R3* mutation is caused by the precise inversion of the *P(lacW)* insert and this correlates with the change of eye phenotype from variegated to red.

In the genetic crosses involving *E1R3*, testing for linkage due to translocations, all four subgroups ($dp^+ e^+$, $dp e^+$, $dp^+ e$, $dp e$) in both sexes were observed (Table 3.2), demonstrating that this mutation was not a translocation involving chromosome 1, 2, or 3.

My examination of the polytene chromosomes of *E1R3/+*, showed no chromosome aberrations when compared with Oregon-R $+/+$ normal control chromosomes. There was no extra chromosome 4 material on other chromosome arms, and there was no distortion or aberration on chromosome 4 either. The mutant chromosome 4 appeared indistinguishable from the normal chromosome 4 control (Figure 3.3).

E2R1 - For *E2R1*, PCR results showed that for primer pairs present on both ends of the *gypsy* insertion, the PCR products were observed, indicating that the *gypsy* insertion was intact. For primer pairs located within the *lacZ* gene, however, no products were amplified,

suggesting there was an internal deletion involving the *lacZ* gene and the two breakpoints of the deletion were located in that region. For the primer pair just outside the deleted region, the PCR product was 4.2 kbp smaller than the expected size, indicating that the deletion was 4.2 kbp in size. I used the PCR primers just outside the deleted region to amplify across the deletion breakpoints and sequence the resulting fragment. Comparing the sequence of the fragment spanning the deletion with that of the undeleted region, the sequence of the fragment spanned from the sequence between the *white* gene and the *lacZ* gene right to the end of *P(lacW)*, missing the 4.2 kbp sequence in between. These results indicated that there was a 4.2 kbp deletion in *P(lacW)* involving *lacZ* sequences but the *white* gene remained intact (Figure 3.6).

Since the *lacZ* sequences in *E2R1* were deleted, the *lacZ* end of the *P(lacW)* insertion couldn't hybridize to the *lacZ* probe. As a result, Southern blot results showed only one original band derived from the *Pci* insertion, for both *AvaI* and *BclI* digestions (Figure 3.6).

In the genetic crosses involving *E2R1*, testing for linkage due to translocations, all four subgroups ($dp^+ e^+$, $dp e^+$, $dp^+ e$, $dp e$) in both sexes were observed (Table 3.2), demonstrating that this mutation was not a translocation involving chromosome 1, 2, or 3.

My examination of the polytene chromosomes of *E2R1/M⁶⁷⁹* (*E2R1/M⁶⁷⁹* polytene chromosomes should have the same phenotype as *E2R1/+* polytene chromosomes, as discussed above), showed no chromosome aberrations when compared with Oregon-R *+/+* normal control chromosomes. There was no extra chromosome 4 material on other chromosome arms, and there was no distortion or aberration on chromosome 4 either. The mutant chromosome 4 appeared indistinguishable from the normal chromosome 4 control (Figure 3.3).

These results indicated that for *E2R1*, there was no second site insertion of *P(lacW)* on chromosome 4 and thus the change of eye phenotype from variegated to red could be caused by the deletion in the *lacZ* gene of the *P(lacW)* insertion.

E1R1 – For *E1R1*, PCR characterization showed that for primer pairs located on both ends of the *gypsy* element and within *P(lacW)*, amplified products were of expected sizes, suggesting that the *gypsy* element was still present, and there was no deletion within the *Pci* insertion (Figure 3.7).

Furthermore, Southern blot results showed extra bands, along with the two original ones derived from the *Pci* insertion, for all four digestions, suggesting that for *E1R1*, there was a second site insertion on chromosome 4. Using the sizes of the extra bands, the mutant

structure was predicted and shown in Figure 3.7, which was caused by a duplication of the *P(lacW)* insertion with some of its distal genomic sequence at the original location.

To examine that possibility, I used primer pairs U-11rpt5 and Jlo12-11rpt5. Primers U and Jlo12 are located in the genomic sequence immediately distal to *P(lacW)*, and primer 11rpt5 is present in the end of *P(lacW)*. Therefore, they are not amplifiable for *E1*, but amplifiable if the *P(lacW)* insertion along with some of its distal genomic sequence are duplicated at the original location (Figure 3.7). PCR results showed a 2.0 kbp band in E1R1 lane but no band in E1 and H₂O lanes for primer pair U-11rpt5, and a 1.3 kbp band in E1R1 lane but no band in E1 and H₂O lanes for primer pair Jlo12-11rpt5 (Figure 3.7). These results suggested that for *E1R1*, the mutation was indeed caused by the duplication of the *P(lacW)* insertion along with some of its distal genomic sequence at the original location.

In the genetic crosses involving *E1R1*, testing for linkage due to translocations, all four subgroups (dp⁺ e⁺, dp e⁺, dp⁺ e, dp e) in both sexes were observed (Table 3.2), demonstrating that this mutation was not a translocation involving chromosome 1, 2, or 3.

My examination of the polytene chromosomes of *E1R1/+*, showed no chromosome aberrations when compared with Oregon-R *+/+* normal control chromosomes. There was no extra chromosome 4 material on other chromosome arms, and there was no distortion or aberration on chromosome 4 either. The mutant chromosome 4 appeared indistinguishable from the normal chromosome 4 control (Figure 3.3).

Therefore, for *E1R1*, the change of the variegated-eye phenotype to red-eye phenotype was caused by reinsertion of *P(lacW)* on chromosome 4, or the duplication of the *P(lacW)* insertion along with some of its distal genomic sequence at the original location, to be more specific.

E1R2 – For *E1R2*, PCR characterization showed that for primer pairs located on both ends of the *gypsy* element and within *P(lacW)*, amplified products were of expected sizes, suggesting that the *gypsy* element was still present, and there was no deletion within the *Pci* insertion (Figure 3.8).

Furthermore, Southern blot results showed extra bands, along with the two original ones derived from the *Pci* insertion (Figure 3.8), suggesting that for *E1R2*, there was a second site insertion on chromosome 4. The new band was only seen for *AvaI* and *BclI* digestions, but not *MscI* and *SnaBI* digestions. These results suggested that *P(lacW)* along with some of its distal and proximal sequences (referred to as the reinserted *P(lacW)* sequences, hereafter) were inserted into a new location. The reinserted *P(lacW)* sequences retained all four *MscI* and *SnaBI* restriction sites, so they generated bands of the same sizes as those of *E1*

control, for *MscI* and *SnaBI* digestions, respectively. In addition, the reinserted *P(lacW)* sequences retained three of the original four *AvaI* and *BclI* restriction sites and the fourth one was from the new location, so they generated one band of a new size and one band of the same size as that of *E1* control, for *AvaI* and *BclI* digestions, respectively.

In the genetic crosses involving *E1R2*, testing for linkage due to translocations, all four subgroups ($dp^+ e^+$, $dp e^+$, $dp^+ e$, $dp e$) in both sexes were observed (Table 3.2), demonstrating that this mutation was not a translocation involving chromosome 1, 2, or 3.

My examination of the polytene chromosomes of *E1R2/+*, showed no chromosome aberrations when compared with Oregon-R *+/+* normal control chromosomes. There was no extra chromosome 4 material on other chromosome arms, and there was no distortion or aberration on chromosome 4 either. The mutant chromosome 4 appeared indistinguishable from the normal chromosome 4 control (Figure 3.3).

Therefore, for *E1R2*, the change of the variegated-eye phenotype to red-eye phenotype was probably caused by the reinsertion of a *P(lacW)* transposon on chromosome 4, but its exact position is unknown.

In summary, the change of the variegated eye phenotype to red eye phenotype can be due to different causes. First, the w^+ phenotype can be caused by the loss of the *gypsy* insertion adjacent to *P(lacW)* (*E1R4*). Second, the precise inversion of *P(lacW)* at the original location converts the variegated phenotype to red phenotype (*E1R3*). Third, a 4.2 kbp deletion involving the *lacZ* gene in *P(lacW)* causes the eye phenotype reversion (*E2R1*). Last, the change of the eye phenotype to red eyes can be due to the reinsertion of a second-site *P(lacW)* on the same chromosome (chromosome 4) (*E1R1* and *E1R2*).

3.2 A screen for mutations that disrupted the mini-white gene of the *E1* and *E2* alleles but retained the ability to trans-silence *Pci*

Both *E1* and *E2* alleles can silence the *Pci* allele in *trans* (Bushey and Locke, 2004). I presume sequences within or adjacent to *P(lacW)* of *E1* and *E2* alleles are required for this *trans*-silencing to occur. As with the first screen, I used $\Delta 2-3$ as the transposase source to mobilize *P(lacW)* of *E1* and *E2* and generate deletions of, within, and adjacent to, *P(lacW)*. Some of these deletions will produce white-eye flies (w^-), so from this screen I recovered w^- mutants. Next, I determined whether the w^- mutants retained the ability to *trans*-silence. If so, I then examined its structure to see which sequences within or adjacent to *P(lacW)* were lost and not required for *trans*-silencing as well as which were retained and thus could be

required for *trans*-silencing. I divided the *w*⁻ mutants up into two main groups: the precise excisions and the internal deletions.

3.2.1 Recovering *w*⁻ mutations

The *P(lacW)* transposon was mobilized by crossing *w*⁻; *Sb e Δ2-3 / TM3 Ser*; *ci¹ ey^R* males to *w*⁻; *E1* (or *w*⁻; *E2 / Dp(2;4) ey^P, ey^P Alp^{MD}*) females (detailed crossing scheme in Figure 2.1). F1 males containing both the $\Delta 2-3$ transposase source and the *E1* or *E2* alleles were crossed to *w*⁻; *dp*; *e*; *ci¹ ey^R* virgin females. In the F2 generation, *w*⁻; *dp/+*; *e/+*; *ci¹ ey^R / Pci* males normally had variegated eyes. Rare mutant white-eye progeny were collected.

I screened about 9,200 flies and recovered 40 white-eye mutant flies (*w*⁻ mutants-Table3.1). The white-eye mutants recovered from the screen above could be caused by the disruption or loss of the *white*⁺ gene in *E1* and *E2* alleles. In such cases, *cis*-silencing can't be assessed. However, such mutations may also affect the *trans*-silencing effect of *E1* and *E2* alleles, and this can be assessed. Each *w*⁻; *dp/+*; *e/+*; *ci¹ ey^R / P(lacW)ci^{Δ2-3}* male with a white-eye phenotype was kept and crossed with *Pci* females. If the *P(lacW)ci^{Δ2-3}* allele retained the *trans*-silencing effect, all the *P(lacW)ci^{Δ2-3} / Pci* flies in the progeny would show a variegated-eye phenotype, instead of the red eye phenotype of *Pci*. Those *P(lacW)ci^{Δ2-3} / Pci* males with a variegated eye phenotype were crossed to *Dp(2;4) ey^P, ey^P Alp^{MD} / Df(4)M101-62f* females to stock the *P(lacW)ci^{Δ2-3}* chromosome in a *w*⁻ background, which will have a *w*⁻ phenotype.

3.2.2 Testing for *trans*-silencing

The 40 *w*⁻ mutants were tested for *trans*-silencing of *Pci*. There were 14 that showed a red eye phenotype, and thus didn't retain any *trans*-silencing ability and were discarded. Four showed a variegated eye phenotype, which was predominantly white, and thus retained what I call "strong" *trans*-silencing ability. The other 22 showed a variegated eye phenotype, which was predominantly red, and therefore retained what I have called "weak" *trans*-silencing ability. Among those 26 that retained some *trans*-silencing ability, 20 were derived from *E1*, named *P(lacW)ci^{E1W1-20}*, and 6 were derived from *P(lacW)ci^{E2}*, named *P(lacW)ci^{E2W1-6}*. They are hereafter called *E1W1-20* and *E2W1-6* in this study (W=white). All 26 mutants were crossed to *Pci* flies and the amount of eye pigment was measured (Figure 3.9) in the mutant/*Pci* male and female progeny.

The eye pigment assay results supported the visual partitioning of the eye phenotypes into "strong" and "weak" groups. As seen in Figure 3.9, four that are labeled "strong" (*E1W10*, *E1W12*, *E1W13* and *E1W18*) have the pigment value below 0.02. The other 22 that are

labeled "weak" have the pigment value mostly within the range between 0.03 and 0.08, rather than the normal, unsilenced value of ~0.14 for *Pci*/+.

In addition, for the 22 mutants that are labeled "weak", I noticed that males have more pigment than females with the same mutant allele. For each *mutant/Pci* genotype, I did pigment assay on samples in triplicate for both males and females, and thus I have three pigment values for both the male and the female groups. I did *t*-tests on each one of the 22 mutants, with the null hypothesis being the means of the pigment values for the male group and the female group are not significantly different.

The degrees of freedom are $2 \cdot (n-1)$ and *n* is 2 in this case, therefore, the degrees of freedom is 2. For each one of the 22 mutants, the mean of the pigment values for the male group is greater than that for the female group (the male-to-female pigment ratio varies from 1.4 to 3.7), therefore the *t*-tests are one-tailed tests, and the critical value of $t_{0.05(2)}$ is 2.920. My observed t_s values vary from 0.65 to 40.01; 4 values (2.00 for *E1W1*, 0.94 for *E1W4*, 0.65 for *E1W6*, and 2.65 for *E1W17*) are less than the critical *t*-value (2.920) and 18 values are greater than the critical *t*-value. Therefore, for the 4 mutant alleles (*E1W1*, *E1W4*, *E1W6* and *E1W17*), the means for the male group and the female group are therefore not significantly different, whereas for the other 18 mutant alleles, the means for the male group and the female group are found to be significantly different. This difference may be caused by an X-linked gene, or a Y-linked gene, or an autosomal gene that is sex-limited in its expression.

I used PCR and DNA sequencing, as described before for the *w** mutations, to determine the molecular structure of the *w** mutants. First, I used PCR primer pairs adjacent to and within the *Pci* insert to test which sequences were deleted. If the sequence between the primer pair was amplifiable but in a smaller size, then there was a deletion in the sequence. Once I determined the approximate extent of the deletion, I used the PCR primers just outside the deleted region to amplify across the deletion breakpoints and sequence the resulting fragment. Comparing the sequence of the fragment spanning the deletion with that of the undeleted region, I can determine the exact size and location of the deletion.

When testing for the presence of the *P(lacW)* insert, three results were possible. First, when both *P* ends were present, we used primers located within *P(lacW)* to test whether there were internal deletions. For two adjacent primers, if one was deleted and the other one was present, then the breakpoint was located in the region between the two primers. If we located two breakpoints, we would sequence the fragment including the two breakpoints and confirm some sequence was deleted between the two breakpoints. If all the primers within *P(lacW)* were present, chances are the *w** mutant phenotype was not caused by an internal deletion.

Second, when only one P end was present, we used primers present within and adjacent to *P(lacW)* to test whether there was a deletion involving one P end and the adjacent genomic sequence. Again, we first used PCR to find the two breakpoints and then sequenced the region spanning the two breakpoints to determine exactly which sequence was deleted.

Third, when neither of the two P ends was present, we used primers located adjacent to, but outside, *P(lacW)* to determine whether the fragment spanning the whole P insert was amplifiable but in a much smaller size due to the excision of *P(lacW)*. If that was the case, we then sequenced the fragment spanning the P excision to see if the excision was precise or not.

3.2.3 Precise excisions of *P(lacW)* from *E1* and *E2* alleles still permits *trans*-silencing

Using methods mentioned above, the structures of the 22 mutant alleles with a weak *trans*-silencing effect were characterized and shown in Figure 3.10. The four mutants with strong *trans*-silencing are described in the next section. For 17 white-eye mutants, the entire *P(lacW)* insert was excised either precisely, or nearly precisely. I examined three of those 17 in more detail (*E1W3*, *E1W5* and *E1W7*) using DNA sequencing to determine whether the excision was, in fact, precise. For *E1W3* and *E1W7*, the excision was precise. For *E1W5*, however, 25 base pairs of the 31 base pair repeats were still present. Nine base pairs belong to the distal 31 base pair repeat of the *P(lacW)* insert, fourteen belong to the proximal 31 base pair repeat, and the middle two could have come from either 31 base pair repeat. Thus, the excision of the *P(lacW)* insert was not precise for *E1W5*.

In summary, since both *E1W3* and *E1W7*, which had the whole *Pci* insert precisely excised, gave a variegated-eye phenotype when heterozygous with the *Pci* allele, I concluded that the *E1* and *E2* alleles do not require any sequences from the *Pci* insert in *cis* to exert some silencing of the *Pci* allele in *trans*. However, this doesn't mean that the presence of *P(lacW)* in *cis* is not involved in this *trans*-silencing.

3.2.4 The degree of *trans*-silencing depends on the amount of the sequence in *P(lacW)* shared by the mutant allele and the *Pci* allele

While all 26 *w* mutants derived from *E1* and *E2* alleles show some level of *trans*-silencing of the *Pci* allele, the degree of the *trans*-silencing varied (Figure 3.9). I correlated the difference in the degree of the *trans*-silencing with the mutant structure.

In the 26 *w* mutants, 22 showed weak *trans*-silencing. Among these 22 mutants, 17 were caused by precise (or almost precise) excision of *P(lacW)* (the excision of the whole 11 kbp *P(lacW)* transgene), four were caused by internal deletions, and the other one was not

caused by precise excision or internal deletion. The four mutants caused by internal deletions are *E1W2*, *E1W9*, *E1W15* and *E2W6* (Figure 3.10). For *E1W2*, *E1W9* and *E1W15*, the deletion didn't involve the 31 base pair repeats at the ends of *P(lacW)*, and the deletion sizes were 9.4 kbp, 10.3 kbp and 10.5 kbp, respectively. *E2W6*, another internal deletion, was caused by a 1.7 kbp deletion within *P(lacW)*. When heterozygous with the *Pci* allele, *E1*-derived precise excisions, *E1W2*, *E1W9* and *E1W15* had the pigment value within the range between 0.03 and 0.08, rather than the unsilenced value of ~0.14 (Figure 3.9). These results suggested that *w⁻* *E1* derivatives had a weak *trans*-silencing effect when the mutations were caused by big internal deletions or precise excision of *P(lacW)*.

In the 26 *w⁻* mutants, four had a strong *trans*-silencing effect. *E1W12*, *E1W13* and *E1W18* all had small internal deletions, 5.4 kbp, 7 kbp and 1.7 kbp, respectively (Figure 3.10). *E1W10* also had a strong *trans*-silencing but was not caused by internal deletions. *E1W12*, *E1W13* and *E1W18*, when heterozygous with the *Pci* allele, had the eye pigment value below 0.02, instead of 0.3–0.8 for the weak *trans*-silencing (Figure 3.9). These results indicated that *w⁻* *E1* derivatives had a strong *trans*-silencing effect when the mutations were caused by small internal deletions of *P(lacW)*.

Taken together, *w⁻* *E1* derivatives caused by internal deletions or precise excision can be divided into two groups by the degree of this *trans*-silencing. *E1*-derived mutants caused by small internal deletions have a strong *trans*-silencing effect, whereas *E1*-derived mutants caused by big internal deletions or precise excision have a weak *trans*-silencing effect. That is, *E1W12*, *E1W13* and *E1W18*, which had a relatively short deletion (5.4 kbp, 7 kbp and 1.7 kbp, respectively), had a strong *trans*-silencing effect; for them, the amount of common *P(lacW)* sequence between the mutant allele and the *Pci* allele was 5.6 kbp, 4 kbp and 9.3 kbp, respectively. On the other hand, *E1W2*, *E1W9*, *E1W15* and the *E1*-derived precise excisions, which had a weak *trans*-silencing effect, had large deletions of, 9.4 kbp, 10.3 kbp, 10.5 kbp, and about 11 kbp, respectively, so the amount of common *P(lacW)* sequence was 1.6 kbp, 0.7 kbp, 0.5 kbp and 0 kbp, respectively. Taken together, the degree of the *trans*-silencing appears to correlate with the amount of *P(lacW)* sequence shared by the mutant allele and the *Pci* allele (Figure 3.11).

Alternatively, instead of a minimal quantity of sequence needed for the strong *trans*-silencing there could be a specific sequence (quality) that is needed for the effect. If so, where could that sequence reside? *E1W12*, *E1W13* and *E1W18*, which have a strong *trans*-silencing effect, retained most of *lacZ* sequence (from 7135 to 10104, using the *P(lacW)* numbering system); whereas *E1W2*, *E1W9*, *E1W15* and the *E1*-derived precise excisions, which had a weak *trans*-silencing effect, retained none of the *lacZ* sequence. Therefore, it is

possible that the strong *trans*-silencing correlates with the presence of specific *lacZ* sequences (from 7135 to 10104, using the *P(lacW)* numbering system) retained in the strong mutant alleles.

3.2.5 The strong *trans*-silencing appears to require a minimal length of common sequence between the silencer and the target instead of the shared reporter gene

The comparisons above show the quantity of transposon sequence shared by the silencer and the target is important for the extent of *trans*-silencing. I further examined whether the quality of the common sequence, like the quantity of it, also plays a role in this *trans*-silencing effect. Since the mini-*white* gene is the reporter gene of the target (*Pci* on the homolog), I examined whether this *trans*-silencing requires the mini-*white* gene to be shared by the target and the silencer (the *E1W* alleles).

As we know, mutant alleles *E1W12*, *E1W13* and *E1W18*, which have a strong *trans*-silencing effect, are caused by internal deletions of *P(lacW)* in *E1* and *E2* alleles. For *E1W18*, about half of the mini-*white* gene of *P(lacW)* is deleted, whereas for *E1W12* and *E1W13*, the mini-*white* gene is completely gone (Figure 3.10). These results suggest that to strongly *trans*-silence the mini-*white*⁺ expression of the *Pci* allele, a minimal quantity of common sequence between the *P* element sequences shared by the silencer and the target, but not the shared mini-*white* sequence itself, between the silencer and the target.

3.2.6 The gypsy containing allele, *ci'*, does not induce *trans*-silencing

As discussed above, the gypsy element of *E1* is responsible for its *cis*-silencing effect. Moreover, the *E1* and *E2* alleles do not require the *P(lacW)* insert in *cis* to exert weak *trans*-silencing of the homologous *Pci* allele. Thus it appears that the gypsy insert alone in the *E1* and *E2* alleles is sufficient for the *trans*-silencing of the *Pci* on the homolog (see *E2W1*). In this regard, the *ci'* mutation is of interest here as it has a gypsy insertion about 600 bp proximal to that of *E2*, and the two gypsy insertions have the same orientation; effectively a chromosome of very similar local structure, but different origin and background. As shown in Figure 3.12, the eye pigment value for *ci'ey^h/Pci* flies was approximately the same as that for *+/Pci* flies, indicating that the gypsy insertion in *ci'* allele alone was not sufficient to induce silencing the *Pci* in *trans* on this chromosome. Nevertheless, in the *w⁺* derivative *E1R4* the gypsy element was lost and a non-silencing phenotype was regained.

Both the *ci'* allele and *E2W1* have a gypsy element insertion and lack a *P(lacW)* insert. The gypsy element in *ci'* is 600 bp proximal to that in *E2W1*, but the two gypsy insertions have the same orientation. The *ci'* allele can't silence the *Pci* allele in *trans* while *E2W1* can.

Therefore, the different location of the two *gypsy* insertions or the different genetic background (chromosome 4) could affect the ability to silence the *Pci* allele in *trans* for the two alleles.

3.2.7 Polytene chromosome squashes for *E1W10* and *E1W17*

Molecular characterization of *w* mutants showed that of all the 26 *w* mutants, only *E1W10* and *E1W17* were not caused by either internal deletions or excision of *P(lacW)*. Thus, these two were further characterized by an examination of their polytene chromosomes. *E1W10* and *E1W17* are both recessive lethal, so they are both stocked over *Dp(2;4)ey^D,ey^DAlp^{no}* (called *ey^D* hereafter). *E1W10/ey^D* males and *E1W17/ey^D* males were crossed to *+/+* (Oregon-R stock) females to generate *E1W10/+* and *+/ey^D* larvae, and *E1W17/+* and *+/ey^D* larvae, respectively. Since the genotype of the larvae could be *E1W/+* or *+/ey^D*, each slide was first compared with the *+/ey^D* slide. Those resembling the *+/ey^D* were discarded, and the *E1W/+* slides were kept for further examination. Three slides for *E1W10/+* and *E1W17/+* were selected, respectively, and they were compared with *w^{ms}* translocation control.

My examination of the polytene chromosomes of *E1W10/+* and *E1W17/+*, showed no chromosome aberrations when compared with Oregon-R *+/+* normal control chromosomes. There was no extra chromosome 4 material on other chromosome arms, and there was no distortion or aberration on chromosome 4 either. The mutant chromosome 4 appeared indistinguishable from the normal chromosome 4 control (Figure 3.13).

3.3 The strong *trans*-silencing may be caused by the formation of a heterochromatic state in the region where *P(lacW)* is located

Since *Su(var)* mutations generally suppress heterochromatic PEV, their impact on *trans*-silencing of *Pci*, if any, will indicate that heterochromatin plays a role in this *trans*-silencing. Therefore, first I examined whether the strong *trans*-silencing of *E1*-derivatives is influenced by a parental origin. Next, I tested whether this strong *trans*-silencing can be suppressed by classic suppressors of heterochromatic PEV.

3.3.1 Strong *trans*-silencing has no parental effect

We may have to take into account the parental source in the testing of my mutations in the *trans*-silencing of *Pci* because the *trans*-silencing effect of the *T-1* cluster of *P-lacZ-white* genes showed a maternal effect (Ronsseray et al, 2001). Thus, I tested whether the strong *trans*-silencing effect of *w* *E1* derivatives was due to parental origin.

E1W10, *E1W18*, *E1W12* and *E1W13* can strongly silence the mini-white (*w*^r) expression of *Pci* on the homolog. I crossed *E1W10/ey^D* males to *Pci/Pci* females to generate *E1W10/Pci* flies, and I also crossed *E1W10/ey^D* females to *Pci/Pci* males (the reciprocal cross) to generate flies of the same genotype, but of different parental origin of the *trans*-silencing allele. This cross, and its reciprocal cross, were also made with *E1W18*, *E1W12* and *E1W13*. Then I measured the amount of eye pigment for *E1W10/Pci*, *E1W18/Pci*, *E1W12/Pci* and *E1W13/Pci* flies derived from the crosses and their reciprocal crosses. As shown in Figure 3.14, for all four mutant alleles the amount of eye pigment was equivalent when the mutant allele was paternal or maternal in origin, suggesting that *trans*-silencing is not influenced by a parental effect. Thus, when I tested other *Su(var)* mutations, the reciprocal cross was not necessary for determining eye pigment and only one cross was made for the eye pigment assay.

3.3.2 Strong *trans*-silencing is sensitive to *Su(var)* mutations.

To test whether classic suppressors of hPEV had an impact on the *trans*-silencing of *Pci*, I crossed in mutants of a classic suppressor, *Su(var)3-7*. Allele *Su(var)3-7^{PS}* was provided by Daniel Bushey. It has a 12 bp deletion in *Su(var)3-7* gene that disrupts its function (Daniel Bushey's doctoral dissertation). When two wild type copies of *Su(var)3-7* are present, the phenotype of strong *trans*-silencing is a predominantly white eye phenotype whereas the phenotype of weak *trans*-silencing is predominantly red. If *Su(var)3-7^{PS}* has an impact on this strong *trans*-silencing, the eye phenotype will change from predominantly white to predominantly red, which can be easily seen by eye before doing the quantitative eye pigment assay.

I crossed *E1W10/ey^D* males to *w; Su(var)3-7^{PS}/TM6B; Pci/Pci* females to generate *w; Su(var)3-7^{PS}/+; E1W10/Pci* flies and measured the eye pigment level. The eye pigment level of *w; E1W10/Pci* flies was below 0.020, whereas that of *w; Su(var)3-7^{PS}/+; E1W10/Pci* flies was above 0.100 (Figure 3.14). Thus, the strong *trans*-silencing of *E1W10* was suppressed by *Su(var)3-7^{PS}*, which is a classic suppressor of hPEV. Similar results were observed for *E1W18*, *E1W12* and *E1W13*. These results suggested that the *trans*-silencing of *E1* and *E2* alleles can be suppressed by a classic suppressor of hPEV, and therefore, the silencing of *w*^r involves proteins known to form heterochromatin.

Table 3.1 Results of screen of mobilized $P(lacW)cf^{Dplac}$

Mutant phenotype	Number of flies screened	Number of mutants					
		Putative mutants		w ⁺ mutants segregating with chromosome 4		w ⁻ mutants showing a trans-silencing effect	
		$P(lacW)cf^{E1}$ (E1) derivatives	$P(lacW)cf^{E2}$ (E2) derivatives	$P(lacW)cf^{E1}$ (E1) derivatives	$P(lacW)cf^{E2}$ (E2) derivatives	$P(lacW)cf^{E1}$ (E1) derivatives	$P(lacW)cf^{E2}$ (E2) derivatives
w ⁺	9,700	45	37	4	1	NA	NA
w ⁻	9,200	30	10	NA	NA	20	6

NA: not applicable.

Table 3.2 Results of genetic crosses testing for the presence of translocations in the five w^+ mutants

	Genotype (eye phenotype)	dp and e Phenotypes				Total
		dp ⁺ e ⁺	dp ⁻ e ⁺	dp ⁺ e ⁻	dp ⁻ e ⁻	
E1R1	E1R1/ci ¹ ey ^R (w ⁺)	7	8	13	11	39
	ci ¹ ey ^R /ci ¹ ey ^R (w)	12	15	10	14	51
E1R2	E1R2/ci ¹ ey ^R (w ⁺)	16	14	19	9	58
	ci ¹ ey ^R /ci ¹ ey ^R (w)	14	19	16	16	65
E1R3	E1R3/ci ¹ ey ^R (w ⁺)	18	18	25	18	79
	ci ¹ ey ^R /ci ¹ ey ^R (w)	20	25	25	21	91
E1R4	E1R4/ci ¹ ey ^R (w ⁺)	28	23	19	25	95
	ci ¹ ey ^R /ci ¹ ey ^R (w)	26	23	12	17	78
E2R1	E2R1/ci ¹ ey ^R (w ⁺)	22	22	17	16	77
	ci ¹ ey ^R /ci ¹ ey ^R (w)	30	22	16	14	82

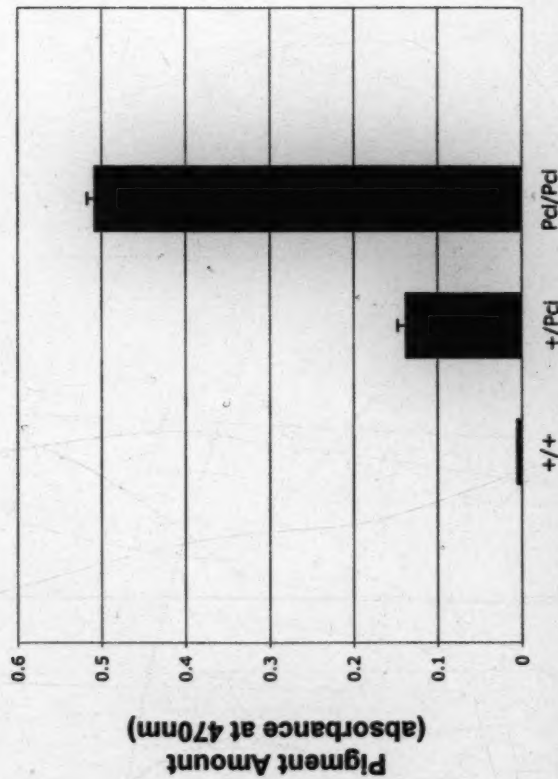


Figure 3.1 Amount of eye pigment varies with dose of *P(lacW)c^{Dplac}*. Flies of the noted genotypes were assayed for eye pigment amount. Eye pigment was extracted from fly heads in groups of 20 and absorbance was measured at 470nm. Three samples were used for each genotype, and mean values (bar) of triplicate samples are reported with the standard error of the mean indicated (thin line). All stocks are in a *white* background, and therefore +/- males had an eye pigment value of 0.005, essentially no pigment. One copy and two copies of *P(lacW)c^{Dplac}* generated increasing, dose dependent levels of eye pigment.

Figure 3.2 The red eye *E1R4* allele has a precise deletion of the *gypsy* insertion and a partial deletion of the distal 0.5 kbp of *P(lacW)*.

A. Summary of PCR analysis of *E1R4*, the distal end of *P(lacW)* (0.5 kbp) is deleted and the *gypsy* insertion (7.5 kbp) is precisely deleted. The diagram is drawn to scale (3 kbp is on the right). The diagram is the structure of parental *E1* allele. The *P(lacW)* construct and the *gypsy* element are inserted between the *ci* gene and *RpS3A* gene. The *P(lacW)* contains genes: *lacZ*, mini-*white* (*white*), *Amp* and *Ori* (abbreviated as *Amp* in the diagram). The direction of the *gypsy* transcript is depicted by the arrows within the element. The bars above the diagram represent the *E1R4* regions that were tested by PCR using primers located at both ends of each bar. A solid bar indicates the PCR product was amplified (the region is intact in the *E1R4* derivative), whereas a clear bar indicates the product is absent because the region is disrupted by a deletion or some other aberration. The PCR results for *E1R4* suggest that *P(lacW)* is partially deleted at the distal end and the *gypsy* element is also deleted. Sequencing results show that the distal end of the *P(lacW)* construct (0.5 kbp) is deleted and the *gypsy* insertion (7.5 kbp) is precisely deleted, as described by the dotted lines. The structure of *E1R4* is also drawn to scale.

B. Restriction map for *E1R4* deduced from Southern blots and PCR results. The diagram is the mutant structure of *E1R4*. It is drawn to scale (3 kbp is on the right). Four types of restriction sites are indicated in the diagram by vertical lines. Different heights of the vertical lines are specific to each restriction site. From top to bottom, they represent *AvaI* digestion, *BclI* digestion, *MscI* digestion and *SnaBI* digestion, respectively. The numbers between the vertical lines indicate the expected size for the restriction band. The dotted lines represent the template DNA that was used to make radioactive probes for the Southern blot analysis. The dotted lines above the *P(lacW)* construct are used for *AvaI* and *BclI* digestions, while the dotted lines beneath the *P(lacW)* construct are used for *MscI* and *SnaBI* digestions. If the *BclI* site in the middle is absent (indicated by X), the new restriction band is 17.3 kbp instead of 10.1 kbp.

C. One-day exposure autoradiographs of Southern blots using restriction enzymes *AvaI*, *BclI*, *MscI* and *SnaBI*. The digested genomic DNA is indicated above each lane. *Pci* genomic DNA is included as a parental control, since it has the original *P(lacW)* construct but not the *gypsy* element and thus is comparable to *E1R4*. The restriction enzyme used is indicated for each set of genomic DNA. The expected sizes are indicated for the bands shown in the autoradiographs. For *BclI* digestion, in *E1R4* lane the 10.1 kbp is not seen whereas the 17.3 kbp band is observed, indicating the *BclI* site (labeled X in B) is absent.

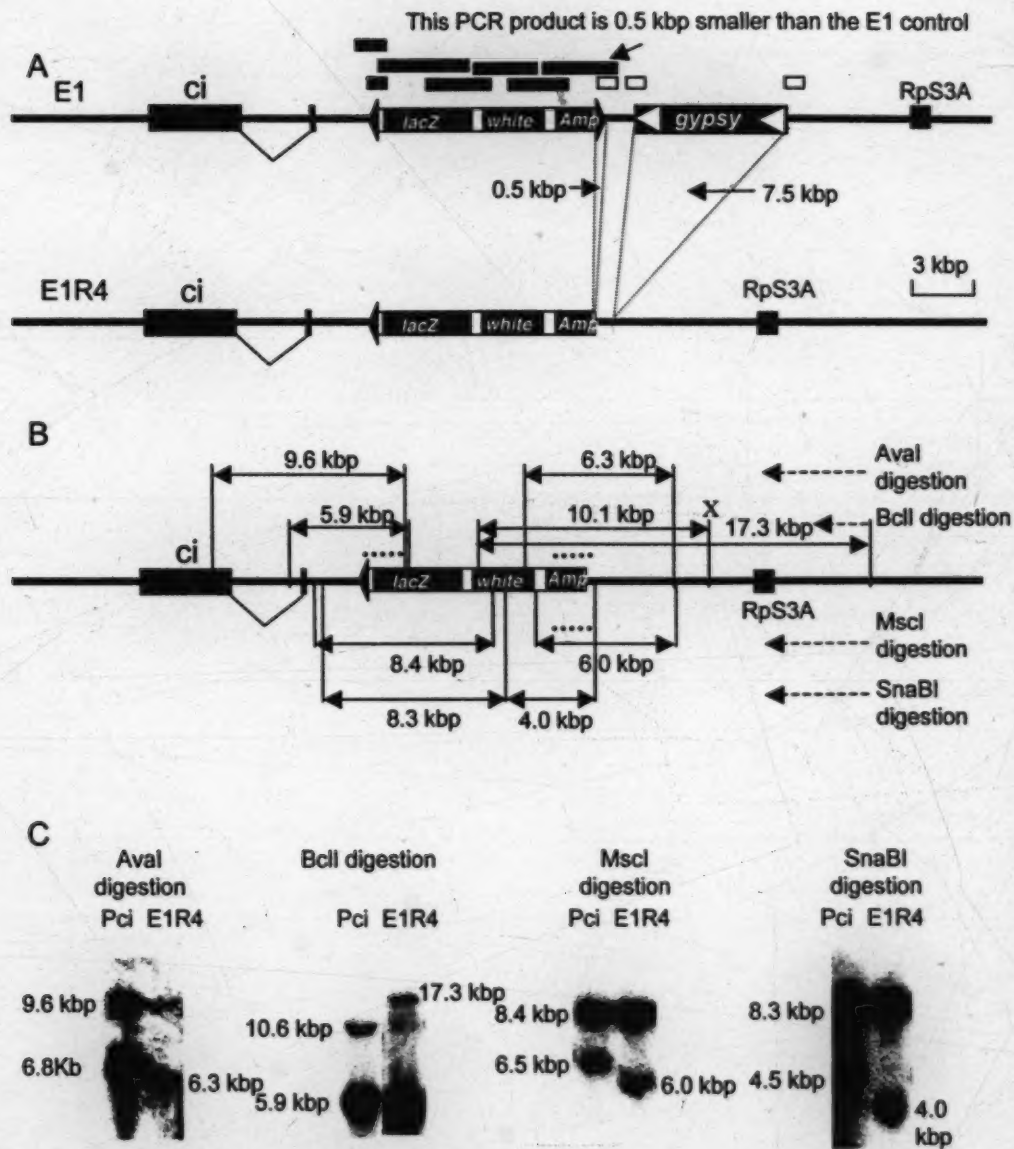


Figure 3.2 The red eye *E1R4* allele has a precise deletion of the *gypsy* insertion and a partial deletion of the distal 0.5 kbp of *P(lacW)*.

Figure 3.3 The polytene chromosome 4 of *E1R1/+*, *E1R2/+*, *E1R3/+*, *E1R4/+*, or *E2R1/M^{57g}* showed no chromosome aberrations when compared with Oregon R *+/+* normal control chromosome 4. The eight pictures are each labeled with a letter as well as the genotype of the larvae from which polytene chromosome squashes were made. From A to H, the genotypes are *+/+*, *ey^D/+*, *w^{m5}/+*, *E1R1/+*, *E1R2/+*, *E1R3/+*, *E1R4/+*, and *E2R1/M^{57g}*, respectively.

A. Polytene chromosomes of *+/+* are shown. Chromosome 4 is indicated by a bracket. Tip represents the tip of chromosome 4, and CC stands for chromocenter. B. The cytologically visible aberration is seen for *ey^D/+* chromosome 4, which is due to a duplication in the middle of chromosome 4. The chromocenter and chromosome 4 tip are both indicated by an arrow. C. For *w^{m5}/+*, the translocated chromosome 4 is seen on the tip of X chromosome, because *w^{m5}* is caused by the translocation involving chromosomes X and 4. X-chromosome and its tip, as well as the translocated chromosome 4 and its tip, are indicated.

D, E, F, G and H. For *E1R1/+*, *E1R2/+*, *E1R3/+*, *E1R4/+*, and *E2R1/M^{57g}*, the mutant chromosome 4 appears indistinguishable from the *+/+* normal chromosome 4 control. Both the chromocenter and chromosome 4 tip are indicated. Since *E2R1* chromosome is stocked over *M^{57g}* chromosome and *M^{57g}* chromosome 4 appears indistinguishable from the *+/+* normal chromosome 4 control, *E2R1/M^{57g}* larvae were used for polytene chromosome squashes.

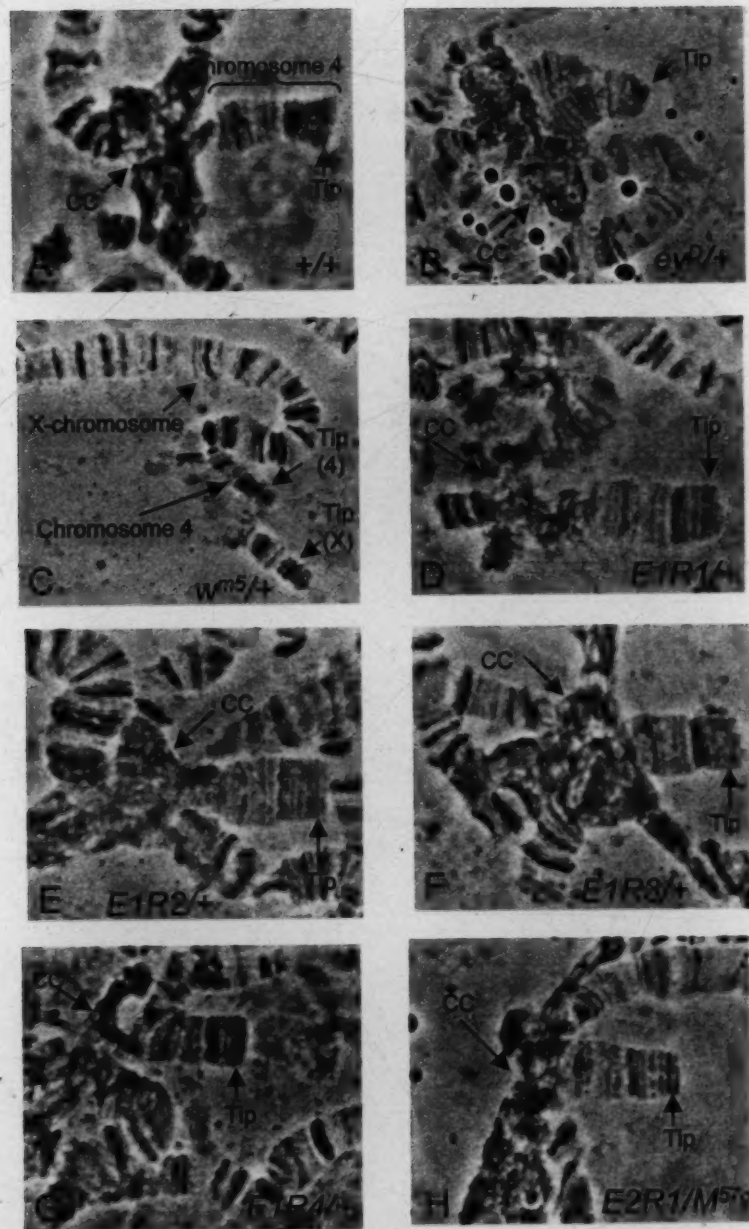


Figure 3.3 The polytene chromosome 4 of *E1R1/+*, *E1R2/+*, *E1R3/+*, *E1R4/+*, or *E2R1/M^{57g}* showed no chromosome aberrations when compared with Oregon R *+/+* normal control chromosome 4.

Figure 3.4 The red eye *E1R3* has an inversion of *P(lacW)* at its original location.

A. Summary of PCR analysis of *E1R3*. The diagram is drawn to scale (3 kbp is on the right). The diagram is the structure of parental *E1* allele. The *P(lacW)* construct and the *gypsy* element are inserted between the *ci* gene and *RpS3A* gene. The *P(lacW)* construct contains genes: *lacZ*, mini-white (*white*), *Amp* and *Ori* (abbreviated as *Amp* in the diagram). The direction of the *gypsy* transcript is depicted by the arrows within the element. The bars above the diagram represent the *E1R3* regions that were tested by PCR using primers located at both ends of each bar. A solid bar indicates the PCR product was amplified (the region is intact in the *E1R3* derivative), whereas clear bar would have indicated the product is absent because the region is disrupted by a deletion or some other aberration. The PCR results for *E1R3* show that there is no deletion within *P(lacW)* and the two breakpoints are present at the two ends of *P(lacW)*, suggesting that *P(lacW)* is inverted. The PCR results also suggest that the *gypsy* element is present.

B. Restriction map for *E1R3* deduced from Southern blots and PCR results. The diagram is the mutant structure of *E1R3*. It is drawn to scale (3 kbp is on the right). Two types of restriction sites are indicated in the diagram by vertical lines. Different heights of the vertical lines are specific to each restriction site. From top to bottom, they represent *AvaI* digestion and *BclI* digestion, respectively. The numbers between the vertical lines indicate the expected size for the restriction band. The dotted lines represent the template DNA that was used to make radioactive probes for the Southern blot analysis.

C. One-day or 4-day exposure autoradiographs of Southern blots using restriction enzymes *AvaI* and *BclI*. The digested genomic DNA is indicated above each lane. *E1* genomic DNA is included as a parental control. The restriction enzyme used is indicated for each set of genomic DNA. The expected sizes are indicated for the bands shown in the autoradiographs. One-day or 4-day exposure is also indicated for *BclI* digestion.

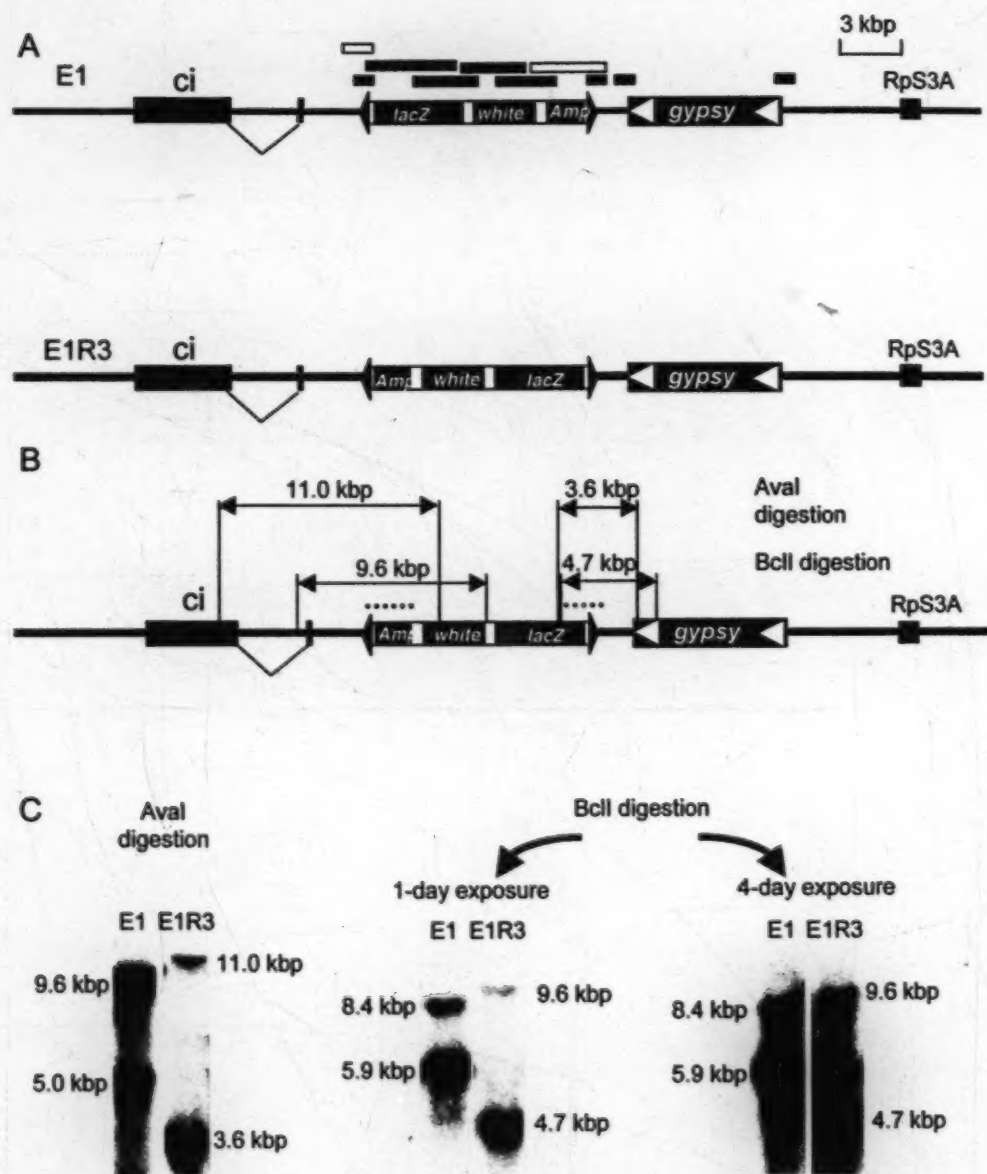


Figure 3.4 The red eye *E1R3* has an inversion of *P(lacW)* at its original location.

Figure 3.5 PCR results confirmed that the red eye *E1R3* is caused by the inversion of *P(lacW)* at its original location.

The structures of *E1* and *E1R3* are drawn to scale (3 kbp is on the right). The diagram is the structure of parental *E1* allele. The *P(lacW)* construct and the *gypsy* element are inserted between the *ci* gene and *RpS3A* gene. The *P(lacW)* construct contains genes: *lacZ*, mini-white (*white*), *Amp* and *Ori* (abbreviated as *Amp* in the diagram). The direction of the *gypsy* transcript is depicted by the arrows within the element. For *E1*, the *P(lacW)* construct has forward primers JS5 and 11668 and reverse primers 20993 and JS1, depicted by arrows. For *E1R3*, the *P(lacW)* construct is inverted, so the primer sites for 20993 and 11668 are changed, as indicated in the diagram. The region between JS5 and 11668 should be amplifiable and produce a 1.0 kbp band for *E1R3*, but not amplifiable for *E1*. The region between 20993 and JS1 should be amplifiable and produce a 0.7 kbp band for *E1R3*, but not amplifiable for *E1*. The presence of the regions between primer pairs JS5-11668 and 20993-JS1 was tested by PCR and gel electrophoresis. The region between primers JS5 and 11668 is present for *E1R3*, indicated by a 1.0 kbp band shown on the 0.8% agarose gel. But the region is absent for *E1* (no band on the gel). *E1* and H_2O lanes are used as positive and negative controls, respectively. The region between 20993 and JS1 is present for *E1R3* but absent for *E1*, suggested by a 0.7 kbp band in *E1R1* lane and no band in *E1* lane.

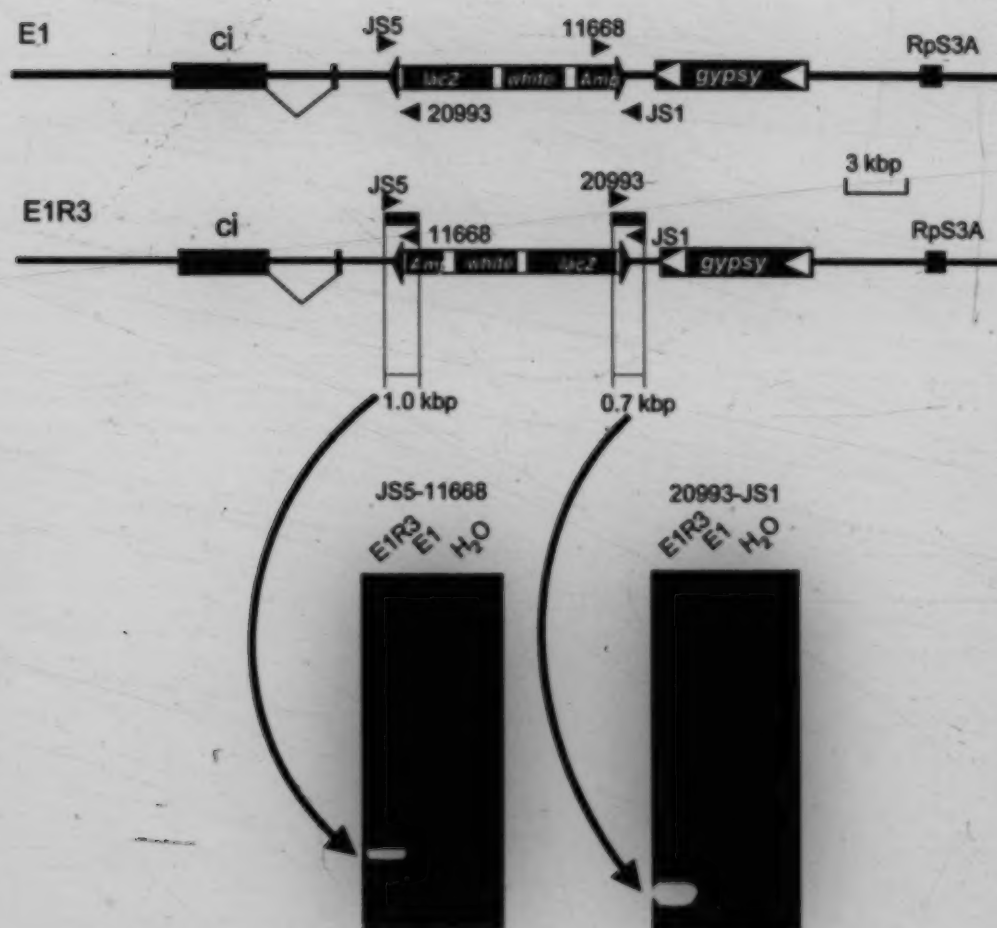


Figure 3.5 PCR results confirmed that the red eye *E1R3* is caused by the inversion of *P(lacW)* at its original location.

Figure 3.6 The red eye *E2R1* allele has a deletion of the 4.2 kbp sequence (containing the whole *lacZ* gene) within *P(lacW)*.

A. Summary of PCR analysis of *E2R1*. The diagram is drawn to scale (3 kbp is on the right). The diagram is the structure of parental *E2* allele. The *P(lacW)* construct and the *gypsy* element are inserted between the *ci* gene and *RpS3A* gene. The *P(lacW)* contains genes: *lacZ*, mini-white (*white*), *Amp* and *Ori* (abbreviated as *Amp* in the diagram). The direction of the *gypsy* transcript is depicted by the arrows within the element. The bars above the diagram represent the *E2R1* regions that were tested by PCR using primers located at both ends of each bar. A solid bar indicates the PCR product was amplified (the region is intact in the *E2R1* derivative), whereas clear bar would have indicated the product is absent because the region is disrupted by a deletion or some other aberration. The PCR results for *E2R1* suggest that the deletion is within the *P(lacW)* construct and involves the whole *lacZ* gene. Sequencing results show that the 4.2 kbp deletion is within *P(lacW)*, and contains the whole *lacZ* gene, as described by the dotted lines. The structure of *E2R1* is also drawn to scale.

B. Restriction map for *E2R1* and *E2*. The structures of *E2R1* and *E2* are drawn to scale (3 kbp is on the right). Two types of restriction sites are indicated in the diagram by vertical lines. Different heights of the vertical lines are specific to each restriction site. From top to bottom, they represent *AvaI* digestion and *BclI* digestion, respectively. The numbers between the vertical lines indicate the expected size for the restriction band. The dotted lines represent the template DNA that was used to make radioactive probes for the Southern blot analysis.

C. One-day exposure autoradiographs of Southern blot using restriction enzymes *AvaI* and *BclI*. The digested genomic DNA is indicated above each lane. *E2* genomic DNA is included as a parental control. The restriction enzyme used is indicated for each set of genomic DNA. The expected sizes are indicated for the bands shown in the autoradiographs.

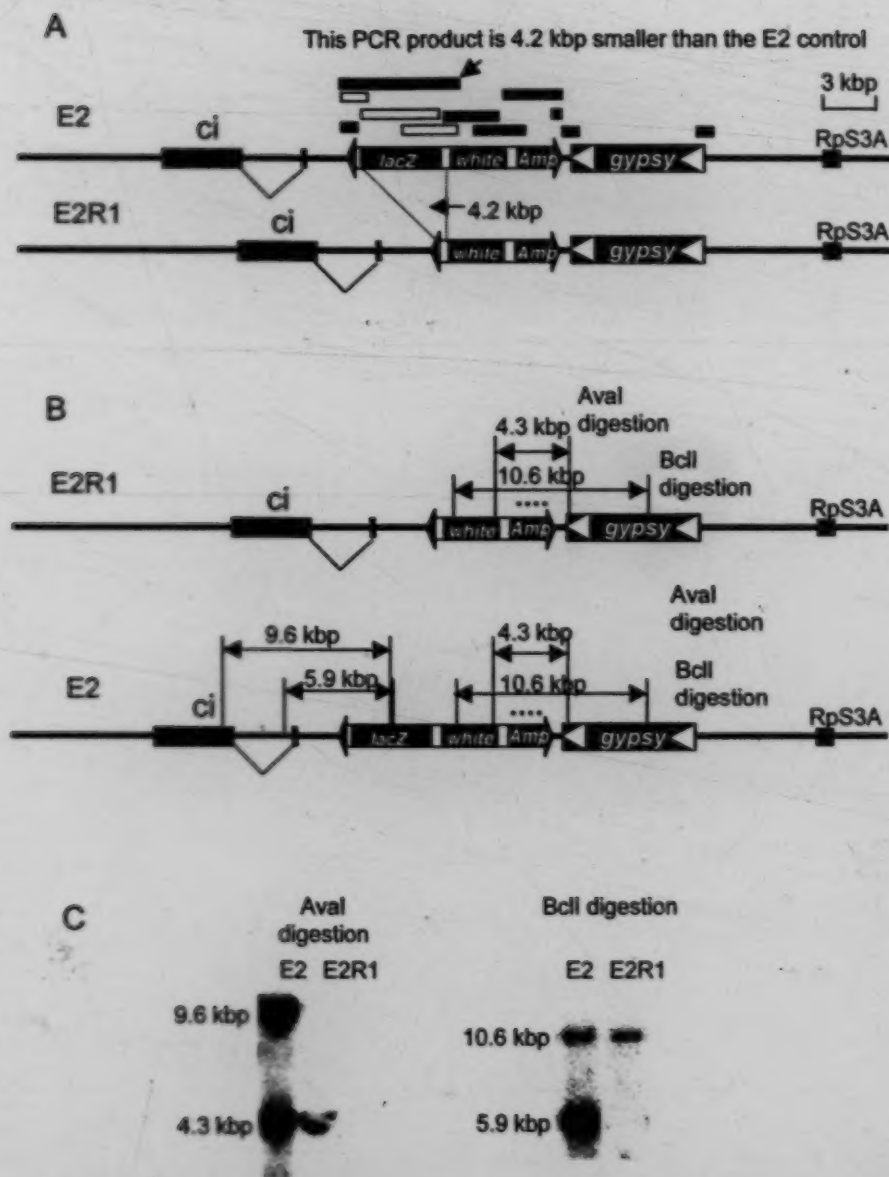


Figure 3.6 The red eye *E2R1* allele has a deletion of the 4.2 kbp sequence (containing the whole *lacZ* gene) within *P(lacW)*.

Figure 3.7 The red eye *E1R1* allele has a tandem duplication of *P(lacW)* along with the distal genomic sequence at its original location.

A. Summary of PCR analysis of *E1R1* The diagram is drawn to scale (3 kbp is on the right). The diagram is the structure of parental *E1* allele. The *P(lacW)* construct and the *gypsy* element are inserted between the *ci* gene and *RpS3A* gene. The *P(lacW)* construct contains genes: *lacZ*, mini-white (*white*), *Amp* and *Ori* (abbreviated as *Amp* in the diagram). The direction of the *gypsy* transcript is depicted by the arrows within the element. The bars above the diagram represent the *E1R1* regions that were tested by PCR using primers located at both ends of each bar. A solid bar indicates the PCR product was amplified (region is intact in the *E1R1* derivative), whereas clear bar would have indicated the product is absent because the region is disrupted by a deletion or some other aberration (Note: none are shown here because all pairs amplified). The PCR results for *E1R1* suggest that the *P(lacW)* construct is intact and the *gypsy* element is present.

B. Restriction map for *E1R1* deduced from Southern blots and PCR results. The diagram is the mutant structure of *E1R1*. It is drawn to scale (3 kbp is on the right). Four types of restriction sites are indicated in the diagram by vertical lines. Different heights of the vertical lines are specific to each restriction site. From top to bottom, they represent *AvaI* digestion, *BclI* digestion, *MscI* digestion and *SnaBI* digestion, respectively. The numbers between the vertical lines indicate the expected size for the restriction band. The dotted lines represent the template DNA that was used to make radioactive probes for the Southern blot analysis. The dotted lines above the *P(lacW)* construct are used for *AvaI* and *BclI* digestions, while the dotted lines beneath the *P(lacW)* construct are used for *MscI* and *SnaBI* digestions.

C. One-day exposure autoradiographs of Southern blots using restriction enzymes *AvaI*, *BclI*, *MscI* and *SnaBI*. The digested genomic DNA is indicated above each lane. *E1* genomic DNA is included as a parental control. The restriction enzyme used is indicated for each set of genomic DNA. The expected sizes are indicated for the bands shown in the autoradiographs.

D. The tandem duplication structure was confirmed by PCR. Some genomic sequence distal to the *P(lacW)* insertion is duplicated along with the *P(lacW)* itself. The genomic sequence distal to the *P(lacW)* insertion contains primer sites U and J1012. The end of *P(lacW)* construct has primer site 11rpt5. The solid bar represents the duplicated genomic region, and the three primer sites within this region are indicated by arrows. The presence of the regions between primer pairs U-11rpt5 and J1012-11rpt5 was tested by PCR and gel electrophoresis. The region between primers U and 11rpt5 is present for *E1R1*, indicated by a 2.0 kbp band shown on the 0.8% agarose gel. But the region is absent for *E1*, suggested by no band on the gel. *E1* and H_2O lanes are used as positive and negative controls, respectively. The region between J1012 and 11rpt5 is present for *E1R1* but absent for *E1*, suggested by a 1.3 kbp band in *E1R1* lane and no band in *E1* lane.

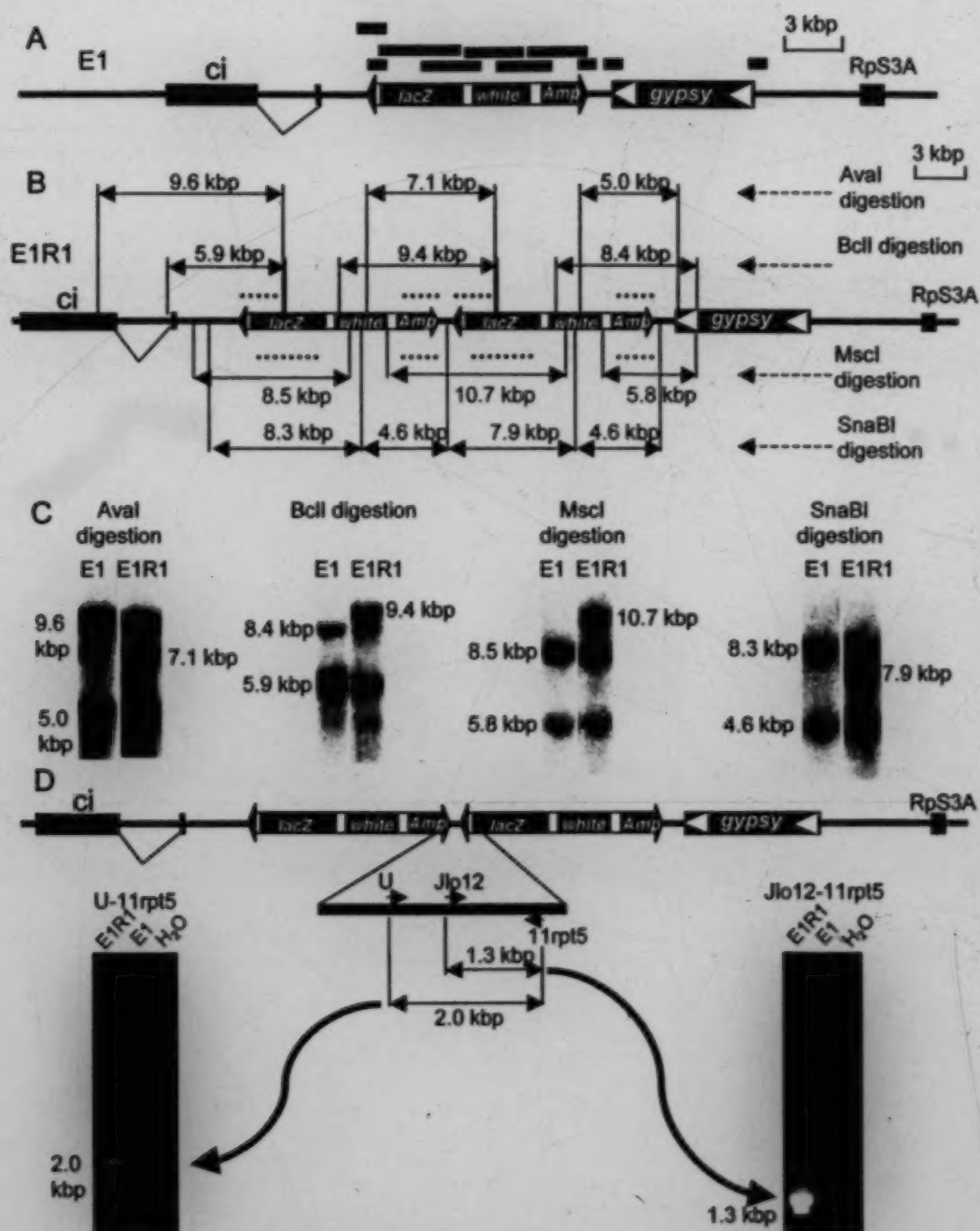


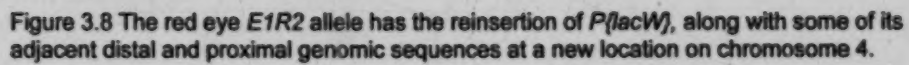
Figure 3.7 The red eye *E1R1* allele has a tandem duplication of *P(lacW)* along with the distal genomic sequence at its original location.

Figure 3.8 The red eye *E1R2* allele has the reinsertion of *P(lacW)*, along with some of its adjacent distal and proximal genomic sequences at a new location on chromosome 4.

A. Summary of PCR analysis of *E1R2*. The diagram is drawn to scale (3 kbp is on the right). The diagram is the structure of parental *E1* allele. The *P(lacW)* construct and the *gypsy* element are inserted between the *ci* gene and *RpS3A* gene. The *P(lacW)* construct contains genes: *lacZ*, mini-white (*white*), *Amp* and *Ori* (abbreviated as *Amp* in the diagram). The direction of the *gypsy* transcript is depicted by the arrows within the element. The bars above the diagram represent the *E1R2* regions that were tested by PCR using primers located at both ends of each bar. A solid bar indicates the PCR product was amplified (the region is intact in the *E1R2* derivative), whereas clear bar would have indicated the product is absent because the region is disrupted by a deletion or some other aberration (Note: none are shown here because all pairs amplified). The PCR results for *E1R2* suggest that the *P(lacW)* construct is intact and the *gypsy* element is present.

B. Restriction map for *E1R2* deduced from Southern blots. The diagram is the mutant structure of *E1R2*. It is drawn to scale (3 kbp is on the right). Four types of restriction sites are indicated in the diagram by vertical lines. Different heights of the vertical lines are specific to each restriction site. From top to bottom, they represent *AvaI* digestion, *BclI* digestion, *MscI* digestion and *SnaBI* digestion, respectively. The numbers between the vertical lines indicate the expected size for the restriction band. The dotted lines represent the template DNA that was used to make radioactive probes for the Southern blot analysis. The dotted lines above the *P(lacW)* construct are used for *AvaI* and *BclI* digestions, while the dotted lines beneath the *P(lacW)* construct are used for *MscI* and *SnaBI* digestions. The dotted vertical lines indicate breakpoints. The sequence between the breakpoints is the *P(lacW)* construct and some of the distal and proximal genomic sequence from its original location (right side). The sequence outside the two breakpoints is sequence from the new location. Thus, the restriction sites between the two breakpoints are exactly the same as those for *P(lacW)* at its original location, whereas the two restriction sites outside the two breakpoints are new (indicated as new - left side).

C. One-day exposure autoradiographs of Southern blots using restrictions enzymes *AvaI*, *BclI*, *MscI* and *SnaBI*. The digested genomic DNA is indicated above each lane. *E1* genomic DNA is included as a parental control. The restriction enzyme used is indicated for each set of genomic DNA. The expected sizes are indicated for the bands shown in the autoradiographs. According to the restriction map for *E1R2* in B, for *AvaI* digestion, the 9.6 kbp band is one copy for both *E1* and *E1R2*, the 5.0 kbp band is one copy for *E1* and 2 copies for *E1R2*, and the new 6.0 kbp band is in *E1R2* lane but not in *E1* lane. For *BclI* digestion, the 5.9 kbp band is one copy for both *E1* and *E1R2*, the 8.4 kbp band is one copy for *E1* and 2 copies for *E1R2*, and the new 6.5 kbp band is in *E1R2* lane but not in *E1* lane. For *MscI* and *SnaBI* digestions, the expected size bands are one copy for *E1* but two copies for *E1R2*.



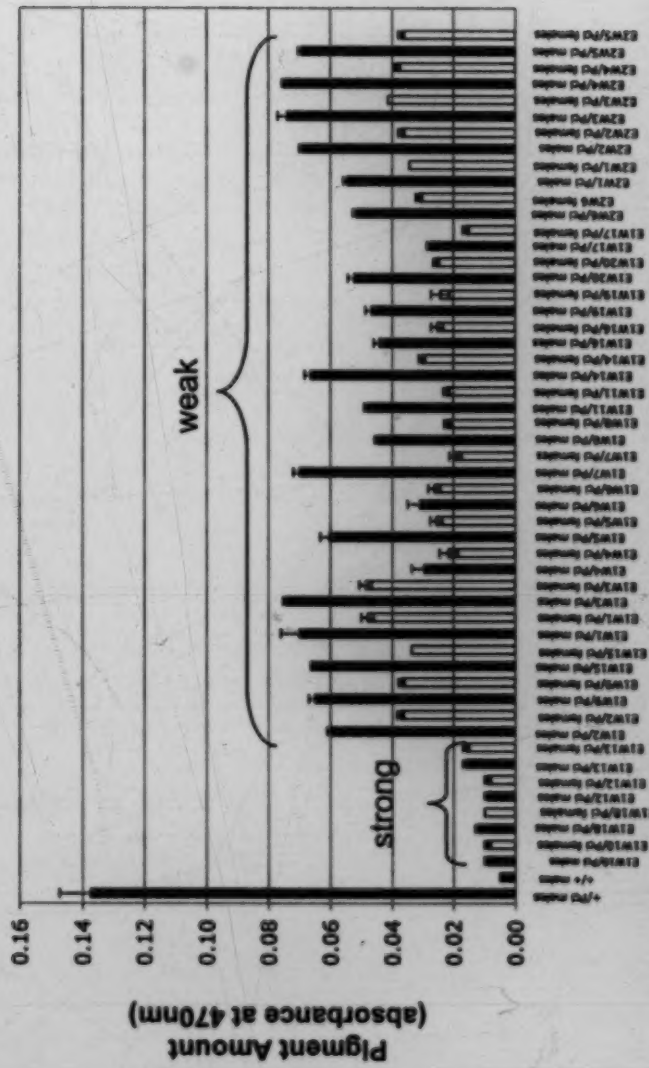


Figure 3.9 The derivatives of $P(lacW)^{cf1}$ (E1) and $P(lacW)^{cf2}$ (E2) alleles can silence the $P(lacW)^{cf3}$ (Pc) construct on the homolog. Flies of the noted genotypes were assayed for eye pigment amount. Eye pigment was extracted from fly heads in groups of 20 and absorbance was measured at 470nm. Three samples were used for each genotype, and mean values (bar) of triplicate samples are reported with the standard error of the mean indicated (thin line). Black bars represent males and white bars represent females. All stocks are in a white background, and therefore +/- males had an eye pigment value of 0.005. In comparison with +/-Pc males, the white-eyed $P(lacW)^{cf1}$ and $P(lacW)^{cf2}$ derivatives can silence the $P(lacW)^{cf3}$ construct in trans. The trans-silencing effect is defined as strong when the pigment value is below 0.02, and weak when the pigment value is between 0.03 and 0.08.

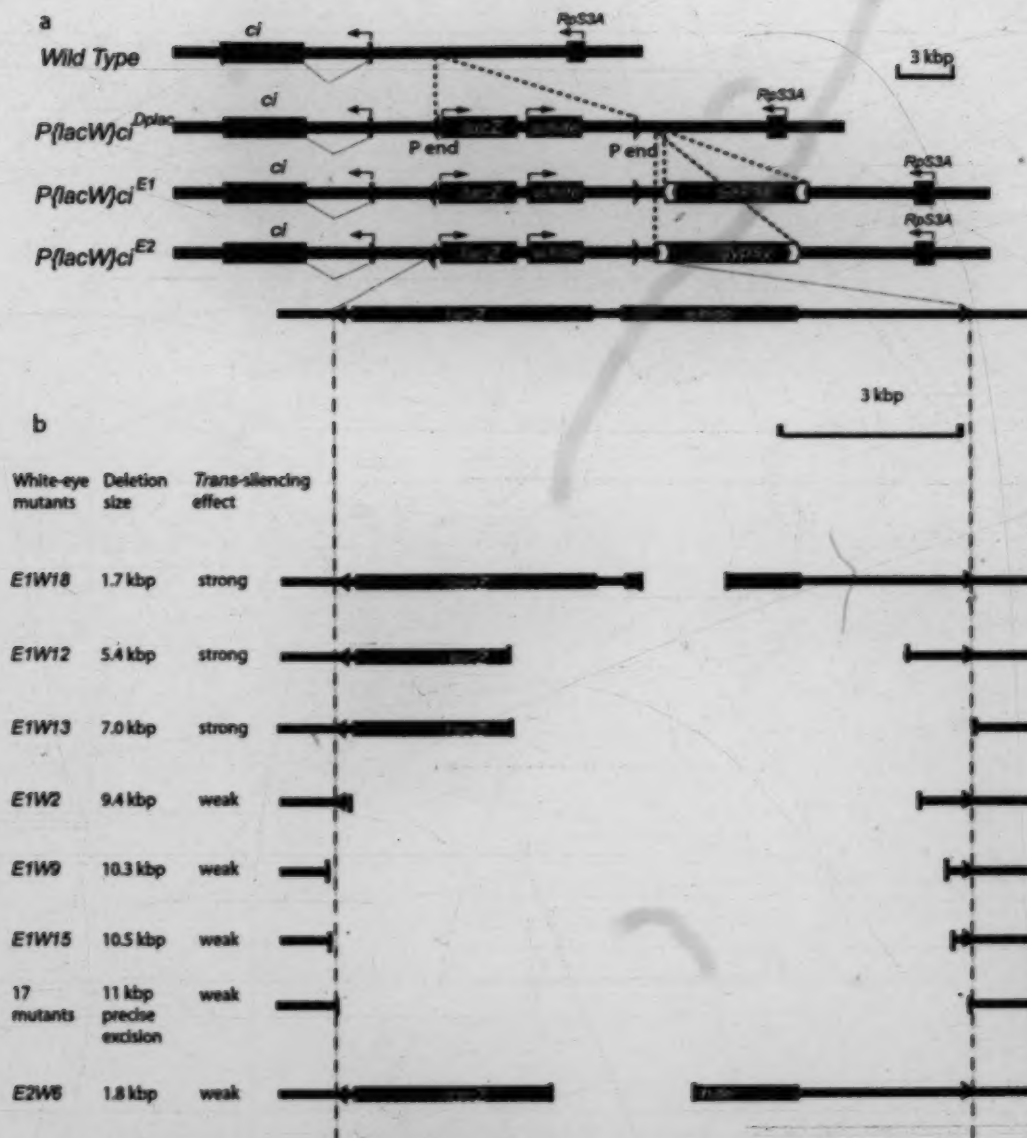


Figure 3.10 Origin and structure of white-eyed mutant alleles recovered from *E1* and *E2*.

A. Diagrams representing wild type chromosome 4, the *Pci* chromosome, the *E1* chromosome and the *E2* chromosome. The chromosome is depicted as a thick line; genes are depicted as solid blocks. Horizontal arrows indicate the promoters of genes; open block arrows on the *gypsy* element indicate orientation of the two LTRs.

B. The structure of each mutant allele is reported. The chromosome is depicted as a thin line; breakpoints caused by a deletion of the chromosome are depicted as vertical lines. The deletion size is listed (if the mutation is caused by a deletion), and the degree of trans-silencing effect is also listed. The two mutants, *E1W10* and *E1W17*, are not caused by deletion or excision of the *Pci* insert, so their structures are not shown in this diagram. To simplify the diagram the mini-white gene's 6 exons are shown as a single block.

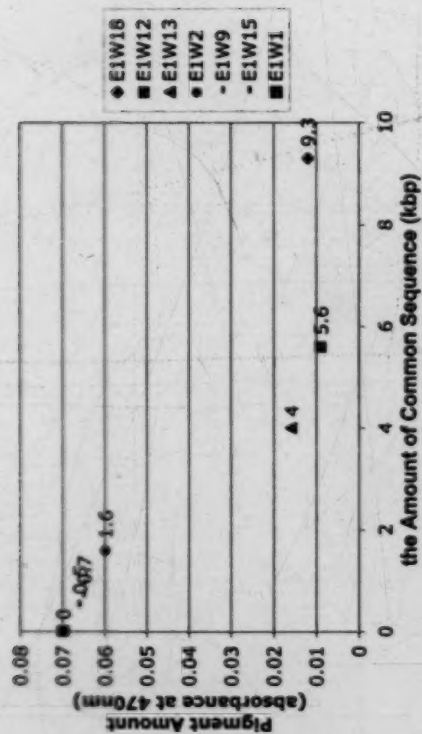


Figure 3.11 The degree of *trans*-silencing depends on the length of *P(lacW)* sequence present in the *P(lacW)_{CE1}* derivatives. The X axis represents the amount of *P(lacW)* sequence, and the Y axis represents pigment amount (absorbance at 470nm). In the graph, each symbol depicts a genotype (listed beside the graph) and is labeled with the length of *P(lacW)* sequence. The *trans*-silencing is weak when the *P(lacW)* sequence is between 0 and 1.6 kbp, whereas it is strong when it is between 4 and 9.3 kbp.

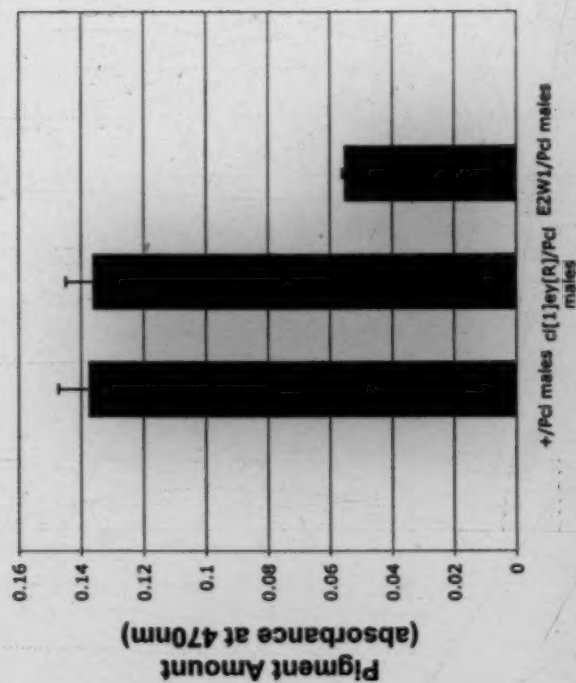


Figure 3.12 The gypsy containing allele, *ci1*, does not induce *trans*-silencing. Flies of the noted genotypes were assayed for eye pigment amount. Eye pigment was extracted from fly heads in groups of 20 and absorbance was measured at 470nm. Three samples were used for each genotype, and mean values (bar) of triplicate samples are reported with the standard error of the mean indicated (thin line). All stocks are in a *white* background. The eye pigment value for *ci1ey(R)/Pd* flies was approximately the same as that for *+/Pd* flies, indicating that the gypsy insertion in *ci1* allele alone was not sufficient to induce silencing of the *Pd* allele in *trans* on this chromosome. The *E2W1/Pd* heterozygote, on the other hand, was able to silence the *Pd* in *trans*.

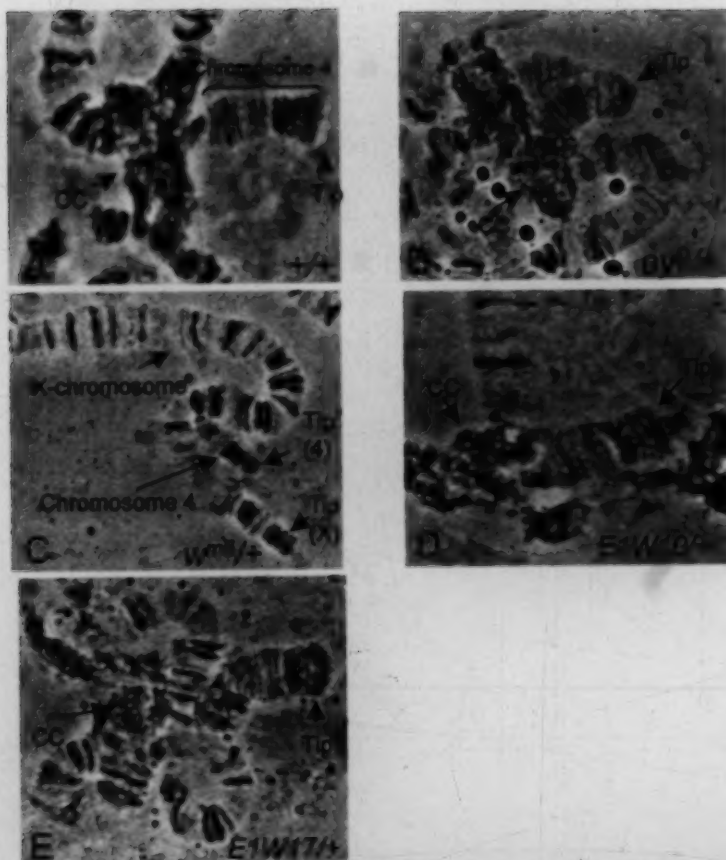


Figure 3.13 The polytene chromosome 4 of *E1W10/+* or *E1W17/+* showed no chromosome aberrations when compared with Oregon R *+/+* normal control chromosome 4. The five pictures are each labeled with a letter as well as the genotype of the larvae from which polytene chromosome squashes were made. From A to E, the genotypes are *+/+*, *ey^D/+*, *w^{ms}/+*, *E1W10/+*, and *E1W17/+*, respectively. A. Polytene chromosomes of *+/+* are shown. Chromosome 4 is indicated by a bracket. Tip represents the tip of chromosome 4, and CC stands for chromocenter. B. The cytologically visible aberration is seen for *ey^D/+* chromosome 4, which is due to a duplication in the middle of chromosome 4. The chromocenter and chromosome 4 tip are both indicated by an arrow. C. For *w^{ms}/+*, the translocated chromosome 4 is seen on the tip of X chromosome, because *w^{ms}* is caused by the translocation involving chromosomes X and 4. X-chromosome and its tip, as well as the translocated chromosome 4 and its tip, are indicated. D and E. For *E1W10/+* and *E1W17/+*, the mutant chromosome 4 appears indistinguishable from the *+/+* normal chromosome 4 control. Both the chromocenter and chromosome 4 tip are indicated.

Chapter 4: Discussion

4 Discussion

4.1 PDS and the *P(lacW)cf* alleles:

The mini-white gene in the *P(lacW)cf^{lac}* enhancer trap insertion is expressed normally in backgrounds lacking *P* elements but is variably silenced in backgrounds containing *P* elements. The mini-white gene shows a variegated phenotype, similar to that of hPEV (Locke et al, submitted). This phenomenon is referred to as *P* element dependent silencing (PDS) (Locke et al, submitted). Bushey and Locke (2004) isolated two derivative alleles of *P(lacW)cf^{lac}* called *P(lacW)cf^{E1}* and *P(lacW)cf^{E2}* (referred to as *E1* and *E2* hereafter), which show a variegated phenotype even in the absence of other *P* elements. Both *E1* and *E2* alleles show a variegated colored eye in M strains, indicating this is *cis*-acting silencing. Moreover, both alleles produce a variegated eye when heterozygous with the parental *P(lacW)cf^{lac}* allele (referred to as *Pci* hereafter), so they also have *trans*-silencing activity (Bushey and Locke, 2004).

Molecular analysis showed that both *E1* and *E2* alleles had a *gypsy* transposable element independently inserted about 1 kbp distal to *Pci* (Figure 1.1). The coincident appearance of the *gypsy* insertions with both *cis*- and *trans*-silencing effects suggested that the *gypsy* elements are, at least in part, responsible for both silencing effects of either *E* allele (Bushey and Locke, 2004).

Thus, in this discussion, I will first discuss the role of the *gypsy* insertion in the *cis*-silencing of *E1* and *E2* alleles. Then I will discuss that the *trans*-silencing of *E1* and *E2* alleles is not only pairing-dependent but also sequence-similarity-dependent. Lastly, I will discuss what molecular mechanisms may be involved in the silencing of *E1* and *E2* alleles.

4.2 The *gypsy* insertion is responsible for the *cis*-silencing of *E1* and *E2* alleles:

The *Pci* allele generates a solid red-eye phenotype in a background lacking *P* elements, whereas both *E1* and *E2* alleles, which have *gypsy* insertions, produce a variegated eye phenotype in a similar genetic background. The pertinent question was whether the *gypsy* insertion alone is sufficient for the *cis*-silencing of *E1* and *E2* alleles. From the $\Delta 2-3$ excision screen, I recovered five red-eyed derivatives of *E1* and *E2* that had the revertant phenotype segregating with chromosome 4. For four of them, the *gypsy* insertion is intact; for the other one, *E1R4*, the whole *gypsy* element (about 7 kbp) is deleted along with the distal end of the *P(lacW)* insert (about 0.5 kbp). Moreover, my Southern blot results suggested that *E1R4* had no second site insertion of *P(lacW)* on chromosome 4. Therefore, in this case, the loss of the

cis-silencing coincided with the loss of the *gypsy* element, suggesting that the *gypsy* element is responsible for the *cis*-silencing of *E1*. Although no *gypsy* deletion mutants were recovered, we assume the mechanism applies to *E2*, too.

4.2.1 The *gypsy* element's insulator activity does not cause the *cis*-silencing of *E1* and *E2*

The insertion of *gypsy* retrotransposons is responsible for mutations that affect genes located throughout the *Drosophila* genome (Modolell et al, 1983). In some it acts as an insulator element and causes mutations by separating enhancers and promoters into different chromatin domains (Gdula et al, 1996). Could the *gypsy* insertion induce the *cis*-silencing of *E1* and *E2* via its insulator function?

Some *gypsy*-induced mutant phenotypes can be reversed by mutations in the suppressor of *Hairy-wing* (*su(Hw)*) gene, indicating that this protein is required for mediating *gypsy* effects on transcription (Lewis, 1981). Mutations in modifier of *mdg4* (*mod(mdg4)*) can affect phenotypes caused by *gypsy* insertion at different loci including yellow (Georgiev and Gerasimova, 1999). At the yellow locus, mutations in *mod(mdg4)* affect enhancers located both distal and proximal to the promoter, instead of only the distal enhancers. This effect is specific for *gypsy*-induced mutations and requires the presence of the *gypsy* element at the yellow locus. A copy of the *gypsy* element lacking the insulator sequences of the *gypsy* element doesn't have this effect, and the *gypsy* insulator alone is sufficient to elicit this effect (Gerasimova et al, 1995). Moreover, *mod(mdg4)* mutations fail to enhance the yellow mutant phenotype when null mutations in the *su(Hw)* gene are present. Thus, *su(Hw)* alone can prevent enhancers from acting in both directions; *mod(mdg4)* appears to change this effect from acting in both directions to acting in one direction. To summarize, *su(Hw)* and *mod(mdg4)* interact with each other and this interaction may be an integral part of the *gypsy* insulator function.

Not only can *gypsy* insertions act as insulators (Gdula et al, 1996; Gerasimova et al, 1995), but *gypsy* insertions at yellow (*y*) can also result in yellow variegation in the absence of the *mod(mdg4)* protein. However, *gypsy* insertions do not normally produce mutations that variegate at other loci (Gdula et al, 1996; Gerasimova et al, 1995). Mutations in *su(Hw)* can suppress both the insulation and variegation in *mod(mdg4)* mutants (Gdula et al, 1996; Gerasimova et al, 1995). Thus, the presence of *su(Hw)* is needed for insulator function.

Bushey and Locke (2004) tested the role of *su(Hw)* in *E1* and *E2*. They tested combinations *su(Hw)²/su(Hw)²*, *su(Hw)²/su(Hw)²* and *su(Hw)²/su(Hw)²* and found that these combinations did not substantially suppress the variegated phenotype of either *E* allele, but

they did suppress known *su(Hw)* dependent mutations (Modolell et al, 1983). Therefore, the *gypsy* element insertions in *E1* and *E2* alleles do not cause the *cis*-silencing of *w*⁺ expression via functioning as an insulator. Instead, since the dose of *Su(var)* loci affects variegation, the silencing may occur by conferring a heterochromatin state to the sequence (Bushey and Locke, 2004).

4.2.2 The *cis*-silencing of *E1* and *E2* alleles may be the result of a heterochromatin state to the region where *P(lacW)* is located

To determine whether the *cis*-silencing of *E1* and *E2* alleles occurs by a change in heterochromatin state, it was necessary to test whether classic suppressors of hPEV had an effect on the *cis*- or *trans*-silencing of *E1* and *E2* alleles. To that end, I tested the effect of a classic suppressor, *Su(var)3-7*, on the eye pigment level of *w*⁺; *E1W10/P(lacW)ci^lPro* flies (Figure 3.14). *E1W10* was suppressed by *Su(var)3-7^{Pro}*, and similar results were observed for *E1W18*, *E1W12* and *E1W13*.

The *Su(var)s*' effect on this *trans*-silencing effect indicates that this protein, and thus heterochromatin, plays a role in this *trans*-silencing. Thus, the *gypsy* element may function by eliciting the formation of a heterochromatic state in the region where *P(lacW)* is located. Exactly how this occurs is not known.

4.2.3 The *gypsy* insertion in *ci^l* allele is not sufficient to induce silencing of the *Pci* in *trans*

As discussed above, the *gypsy* element of *E1* appears responsible for its *cis*-silencing effect. Moreover, *E1* and *E2* alleles do not require the *P(lacW)* insert in *cis* to exert some *trans*-silencing of the *Pci* allele. The significant question is whether the *gypsy* insert alone in *E1* and *E2* alleles can initiate the *trans*-silencing of the *Pci* allele. I examined this question by testing the *ci^l* allele, which has a *gypsy* insertion near that in *E1* and *E2* alleles but no *P(lacW)* insertion on chromosome 4. The *ci^l* allele is also in a different genetic background than the *Pci* allele. The *gypsy* insertion of *ci^l* is about 600 bp proximal to that of *E2*, and the two *gypsy* insertions have the same orientation. My results showed that *ci^l*, with its *gypsy* insertion in the same general region as both *E1* and *E2* alleles, was insufficient to cause silencing the *Pci* in *trans*.

On the other hand, in the *w*⁺ derivative *E2W1*, which had the *P(lacW)* insert excised and only the *gypsy* element of *E2* was left, can still silence the *Pci* allele in *trans*. Comparing the *gypsy* insertion in *ci^l* allele with that in *E2W1*, they have the same orientation, but the *ci^l* *gypsy* insertion is about 600 bp proximal to *E2W1* *gypsy* insertion. The difference in the

trans-silencing ability of *ci^f* and *E2W1* can be explained in two ways. First, it could be due to the difference in the locations of the two *gypsy* insertions in *ci^f* and *E2W1*. Second, the different genetic backgrounds of the *ci^f* allele and *E2W1* allele may be the reason why one can not *trans*-silence *Pci* whereas the other can.

4.3 The *trans*-silencing of *E1* and *E2* alleles is pairing-dependent:

In *Drosophila*, the pairing of homologous chromosomes during interphase can influence gene expression (Duncan, 2002). E.B. Lewis first detected these influences within the bithorax complex (BX-C) and used the term transvection to describe them (Lewis, 1978). Transvection can occur by the action of enhancers or silencers in *trans*, or by the spreading of position effect variegation from rearranged chromosomes to paired unrearranged chromosomes (Duncan, 2002). There are three systems described in the literature that are relevant here. At *yellow* (*y*) locus, it is the enhancers that act in *trans*. For *brown* (*bw*) and *hsp70-white*, it is the silencers that act in *trans*. A comparison of these three systems, with *E1* and *E2* alleles, helps to understand what mechanisms may be involved in the *trans*-silencing of *E1* and *E2* alleles.

4.3.1 Comparing transvection at the *yellow* (*y*) locus with the *trans*-silencing of *E1* and *E2* alleles

Transvection at the *yellow* (*y*) locus was first demonstrated for the *y²* allele (Lindsley and Grell, 1968). In the *y²* allele, a *gypsy* element is inserted at -700 bp from the transcription start site, which separates and uncouples distal wing and body enhancers from the promoter. Consequently, *y²* flies have a mutant yellow cuticle in the wing and body. Geyer et al (1990) characterized a number of *yellow* mutant alleles that can complement *y²*, and give wing and body a normal pigment phenotype. They found that all complementing alleles have the wing and body enhancers, although the *y* promoter was deleted or damaged, suggesting that it is the wing and body enhancers that act in *trans* and drive transcription on the *y²* promoter in the wing and body cuticle (Geyer et al, 1990). *ysm* is derived from *y²* and deletes part of *gypsy*, the *y* promoter, and 5' part of the transcribed region. *ysm* can complement *y²*, but does not complement *y²* transgenes inserted at ectopic locations. This indicates that complementation of *ysm* and *y²* appears to be chromosome-pairing-dependent (Geyer et al, 1990).

In our *trans*-silencing system, *E1* and *E2* alleles can *trans*-silence *P(lacW)* on the homologue. Bushey and Locke (2004) found that this *trans*-silencing was lost when the chromosome 4 containing *P(lacW)* was translocated to another chromosome. Therefore, the

trans-silencing of *E1* and *E2* alleles is also sensitive to chromosome pairing, like that shown in the *y* case.

4.3.2 Comparing the *trans*-silencing of the *bw*⁺ gene and the *hs-white* gene with the *trans*-silencing of *E1* and *E2* alleles

Some *Drosophila* genes can exhibit the action of silencers in *trans* (Duncan, 2002), which can be called *trans*-silencing. When the eye color gene *brown* (*bw*) is placed next to heterochromatin (*bw*⁺ rearrangements), the rearrangements can variably silence not only the *bw*⁺ gene in the rearranged homolog but also the one in the normal homolog to which it is paired (Henikoff and Dreesen, 1989). Since *bw*⁺ transgenes located at ectopic locations are not suppressed by these rearrangements, the silencing of *bw*⁺ in *trans* is pairing-dependent (Dreesen et al, 1991). When the *bw*⁺ gene contained in a *bw*⁺ rearrangement was excised by *P*-element mobilization, silencing of the *bw*⁺ gene on the homolog (in *trans*) was still observed. This suggested that *trans*-silencing does not require the presence of the *bw*⁺ gene in *cis* to the rearrangement (Dreesen et al, 1991). These results indicate that *bw*⁺ is sensitive to repression by heterochromatin and PEV can occur in *cis* as well as in *trans* (Duncan, 2002).

An *hsp70-white* transposon inserted at cytogenetic region 82C produces uniform pigmentation in fly eyes (Martin-Morris et al, 1997). Four different variegating alleles of this *hs-white* transgene were generated by X-ray mutagenesis, and they can *trans*-silence the normally nonvariegating *hsp70-white* transgene on a non-rearranged homolog (Martin-Morris et al, 1997). For three of them (*hs-white*[82C]^{var1}, *hs-white*[82C]^{var2} and *hs-white*[82C]^{var3}), when heterozygous with this nonvariegating *hsp70-white* transgene, the 82C region appears normally paired in polytene chromosomes; for the other one (*hs-white*[82C]^{var4}), only a minor distortion was observed. Moreover, these variegating alleles didn't affect expression of *hs-white* transposon located elsewhere in the genome, which indicated that *trans*-silencing only acts on loci that are paired on the homolog. When the *hs-white* gene in *cis* to the heterochromatic lesion in the variegating alleles was excised by *P*-element mobilization, the excision alleles can still *trans*-silence the parental *hs-white* allele (Martin-Morris et al, 1997). Thus, in both the *bw*⁺ and in these variegating *hs-white*[82C] mutations, the variegating locus is not needed in *cis*.

In our study, *E1* and *E2* can *trans*-silence the *P(lacW)* transgene. Like the *hs-white* gene and the *bw*⁺ gene, *P(lacW)* in *cis* is not required for that *trans*-silencing to occur, however, we show that its presence facilitates the *trans*-silencing that does occur.

To sum up, the sensitivity of different genes to chromosome pairing disruptions is different (Martin-Morris et al, 1997). Rearrangements involving *brown*, although disrupting chromosome pairing, do not result in disrupted pairing of *brown* alleles and *trans*-silencing still occurs (Henikoff and Dreesen, 1989; Dreesen et al, 1991). However, if *white* variegating alleles are caused by gross chromosome rearrangements, chromosome pairing is disrupted when they are heterozygous with *white*⁺, and there is no *trans*-silencing effect (Spofford, 1976). For *hs-white*, lesions leading to *white* variegation of *white* minimally disrupt the linear order of genes on the chromosomes and thus permit close homologous pairing (Martin-Morris et al, 1997). Thus, for this *trans*-silencing, the disruption of chromosome pairing interrupts the pairing of the two alleles involved in the *trans*-silencing, which in turn disrupts the *trans*-silencing. The same mechanism may apply to *Pci*. *E1* and *E2* alleles can *trans*-silence the *P(lacW)* on the homologue. This *trans*-silencing was lost when the chromosome 4 region containing the *P(lacW)* insert was translocated onto other chromosomes. The translocation disrupted chromosome pairing, which disrupted the pairing of the two alleles and thus the *trans*-silencing.

4.4 The shared *P(lacW)* sequence facilitates the ability of *E1* and *E2* alleles to *trans*-silence the *Pci* allele:

Trans-silencing always involves two elements. The first can be called a transmitter, and sends the silencing signal. The second, called a receiver, converts the signal to silence a gene. In *trans*-silencing signaling how is the communication made?

In the *trans*-silencing of *NA-P(1A)*, sequence similarity between the transmitter and the receiver is important. *NA-P(1A)* is a defective *P* element at cytogenetic region 1A on the X-chromosome and is missing the 5' 871 bp (Marin et al, 2000). It can not repress a classical *P-lacZ* construct (*BQ16*), where only 233 bp *P* element sequence is shared by the defective *P* element (*NA-P(1A)*) and the *P* transgene (*BQ16*). *NA-P(1A)* can repress the *PLH-3 P-lacZ* construct, where 1.8 kbp *P* element sequence is shared by the defective *P* element and the *PLH-3* transgene. Thus, the ability of *NA-P(1A)* to silence a *P* transgene in *trans* appears to require a minimal length of common sequence between the two *P* sequences (Marin et al, 2000). No chromosome pairing is proposed here, in this *trans*-silencing.

In our study, the sequence within the *P(lacW)* shared by the weak *trans*-silencing alleles and the *Pci* allele is between 0 kbp and 1.6 kbp; the sequence within *P(lacW)* shared by the strong *trans*-silencing alleles and the *Pci* allele is between 4 kbp and 9.3 kbp. Therefore, greater *trans*-silencing is linked to more shared *P(lacW)* sequences, and the strong *trans*-

silencing requires a minimal length of the shared sequence within *P(lacW)* between the strong *trans*-silencing alleles and the *Pci* allele.

4.4.1 The shared reporter gene (mini-*white*) is not required for the ability of *E1* and *E2* alleles to *trans*-silence the *Pci* allele

The shared *P(lacW)* sequence between the transmitter and the receiver can play a crucial role in *trans*-silencing (Ronsseray et al, 2001). In the *P-Co-1* construct, *lacZ* is not fused in frame with the 5' P sequence but is driven by an ovarian tumor promoter, a promoter that is active in the germline of both sexes. It is strongly expressed in the nurse cells in females that have no P sequence (Ronsseray et al, 2001). *P-Co-1* can be silenced by a *P-lacZ* (*P-1152*) but not by complete P elements (*Lk-P(1A)*) lacking the *lacZ* gene. Therefore, the common 800 bp of the P sequence present in both *P-Co-1* and *Lk-P(1A)* are not sufficient to elicit *trans*-silencing. These data suggest that the repression of *P-Co-1* by *P-1152* is caused not only by the 800 bp P sequence shared by *P-Co-1* and *P-1152*, but also by the *lacZ* sequence shared by them (Ronsseray et al, 2001).

In our study, the expression of mini-*white* gene within the *P(lacW)* was examined. We found *w*⁻ mutant alleles (*E1W12* and *E1W13*), with the whole mini-*white* gene deleted, can still strongly silence *white*⁺ expression from the homolog. This indicates that the presence of mini-*white* gene in *w*⁻ mutant alleles is not required for *trans*-silencing the mini-*white*⁺ gene on the homolog. Therefore, the reporter gene shared by the transmitter and the receiver is not required for the strong *trans*-silencing. The presence of the reporter gene, however, can add to the length of the shared *P(lacW)* sequence. That in turn can increase the extent of *trans*-silencing, since greater *trans*-silencing is dependent upon more shared *P(lacW)* sequence.

4.4.2 Two models to explain the strong *trans*-silencing alleles

There are two, non-mutually exclusive models to explain how the mutant alleles strongly *trans*-silence the *white* gene on the *Pci* homologue.

The first model (quantity) is that strong *trans*-silencing depends on the amount of *P(lacW)* sequence in the mutant allele. The weak *trans*-silencing alleles have less than 1.6 kbp of the *P(lacW)* sequence shared with *Pci*, while the strong *trans*-silencing alleles share between 4 kbp and 9.3 kbp. Therefore, strong *trans*-silencing is linked to greater than 4.0 kbp of shared *P(lacW)* sequences.

The second model (quality) is that strong *trans*-silencing depends on a specific sequence within *P(lacW)* in the mutant allele. Since the *lacZ* sequence is completely (or almost completely) deleted within the weak *trans*-silencing alleles while they are partially retained the

strong *trans*-silencing alleles, strong *trans*-silencing could correlate with the presence of a specific *lacZ* sequence.

These two models are not mutually exclusive, and either model cannot be ruled out at this point. Thus, more work is needed to elucidate which model is involved in this *trans*-silencing.

4.5 Molecular mechanisms of the *trans*-silencing of *E1* and *E2* alleles:

Both *E1* and *E2* alleles have a *gypsy* element inserted about 1 kbp distal to *P(lacW)*, and both appear to silence by a common mechanism. The *trans*-silencing of both *E1* and *E2* alleles can be suppressed by classic modifiers of hPEV (*Su(var)205* alleles or *Su(var)3-7* alleles) and an extra Y-chromosome, all of which suppress hPEV (Bushey and Locke 2004). This suggests that the *trans*-silencing is caused by the formation of a heterochromatic state in the region where the *P(lacW)cf^{Fluor}* construct is located.

4.5.1 Three putative models for *trans*-silencing of *E1* and *E2* alleles

In general, *trans*-silencing can occur at the transcriptional, or post-transcriptional level. At present, there are several models. The first two models described here propose that the transmitter sequence could recognize the receiver(s) at the DNA level by scanning the genome (Matzke et al, 1994). This scanning would lead to pairing of the transmitter sequence and the receiver sequence (Ronsseray et al, 2003). In the first model, the silencing sequence is heterochromatinized, the pairing of the transmitter sequence and the receiver sequence would result in *trans*-heterochromatinization of the receiver (Ronsseray et al, 2003). In the second model, the pairing of the transmitter sequence and the receiver sequence would move the receiver to the compartment of the nucleus where heterochromatin is localized (Csink and Henikoff, 1996; Demburg et al, 1996).

In a third model, the silencer sequences would produce aberrant RNA (prematurely terminated or processed inaccurately) or double-stranded RNA to induce RNA interference and degrade homologous RNAs (Ronsseray et al, 2003).

4.5.2 The first model may apply to the *trans*-silencing of *E1* and *E2* alleles

As mentioned above, *hs-white*[82C], a transposon with *hsp70-white* inserted at 82C, produces uniform pigmentation in fly eyes, and four different variegating alleles of this *hs-white* transgene generated by X-ray mutagenesis can *trans*-silence this nonvariegating transgene (Martin-Morris et al, 1997). The variegating-inducing lesions in these four alleles minimally disrupt the linear order of genes on the chromosomes, and therefore, they still

permit homolog pairing. The *trans*-silencing of these variegating alleles can be suppressed by *Su(var)* mutations, indicating that heterochromatin plays a role in this *trans*-silencing. The situation is different when *white* rearrangements of the endogenous *white* gene include PEV alleles. When heterozygous with *white*⁺, these *white* PEV alleles grossly disrupt homolog pairing and thus they can not silence the *white*⁺ gene on the homolog (Spofford, 1976). Therefore, the *trans*-silencing of the *white* gene requires close pairing of homologues. The model for this chromosome-pairing-dependent *trans*-silencing could be that the transmitter sequence is heterochromatinized and the close pairing of the transmitter and receiver sequences would result in *trans*-heterochromatinization of the receiver allele.

Bushey and Locke (2004) proposed that the P element dependent silencing of the *P(lacW)* is probably due to a local change in the chromatin state. The *gypsy* element inserted in the same region about 1 kbp distal to *P(lacW)*, together with *P(lacW)*, may also initiate the formation of a heterochromatic state in this region, which can silence both the *P(lacW)* in *cis* and, because of pairing, the one in *trans*. Thus, the transmitter sequence itself is heterochromatinized. Moreover, the length of the sequence shared by the *P(lacW)* of *w*⁻ mutant alleles and that of the *Pci* allele can affect the extent of the *trans*-silencing of those *w*⁻ mutant alleles. Therefore, it is likely that the length of the sequence shared by the transmitter sequence and the receiver sequence influences the pairing of them. And that may have an impact on the *trans*-heterochromatinization and thus the *trans*-silencing of the target.

4.5.3 The second model does not apply to the *trans*-silencing of *E1* and *E2* alleles

When the eye color gene *brown* (*bw*) is placed next to heterochromatin (*bw*⁺ rearrangements), the rearrangements can variably silence the *bw*⁺ gene in the rearranged homolog and the one in the normal homolog (Henikoff and Dreesen, 1989). The *bw*-Dominant (*bw*^D) allele is caused by an insertion of 1-2 Mb of heterochromatin into the *bw* gene. When rearrangements move the *bw*^D allele further from the coalesced heterochromatic regions of the genome (the chromocenter), the *trans*-silencing of *bw*^D is suppressed; when rearrangements bring *bw*^D closer to the chromocenter, the *trans*-silencing is enhanced (Talbert et al, 1994). Since the extent of *trans*-silencing correlates with the frequency at which *bw*^D and its paired *bw*⁺ allele become localized to the chromocenter, it suggests that *trans*-silencing is due to the forced relocation to the heterochromatic compartment (Csink and Henikoff, 1996; Dernburg et al, 1996). The ability of *bw*^D to *trans*-silence *bw*⁺ in some drastic rearrangements indicates that these rearrangements may disrupt chromosome pairing but do not result in unpairing of the two *bw* alleles (Henikoff and Dreesen, 1989).

For *E1* and *E2* alleles, the *trans*-silencing was lost when the chromosome 4 containing *P(lacW)* was translocated onto other chromosomes. The translocation disrupted chromosome pairing, which disrupted the pairing of the two alleles and thus the *trans*-silencing. Therefore, this second model may not apply to our case.

4.5.4 The third model may apply to the *trans*-silencing of *E1* and *E2* alleles

Pal-Bhadra et al (1997) studied three insertions of a transgene carrying the promoter region and the eye enhancer of *w* driving the expression of the *Adh* structural gene. When flies containing two or more of these inserts were examined, expression was suppressed rather than increasing in proportion to the added dose. Furthermore, the expression of the endogenous *Adh* gene was also suppressed. The level of *Adh* transcripts decreases progressively with the increased transgene dosage. This suggests the absence of a threshold over which RNA degradation is initiated, while this threshold is important for the post-transcriptional model. The suppression is greater for homozygotes for each insertion than for any pair-wise combinations of insertions. This indicates that homolog pairing has an effect on this process, whereas the effect of homolog pairing is not predicted in the post-transcriptional model. Together, these results suggest that the suppression of these transgenes occurs at the transcriptional level.

When full-length *Adh* transgenes were examined, the suppression is much weaker and post-transcriptional, and clearly occurs by an RNAi-based mechanism. Small interfering RNAs are detected, which are a component of RNAi pathway. In addition, *piwi* mutations, which block RNAi, largely eliminate the suppression. Since homozygous insertions of the *w/Adh* transgenes show greater suppression than combinations of two distant insertions, Birchler et al argued that DNA-DNA pairing must be involved in this transcriptional silencing (Birchler et al, 2000). However, the fact that the transcriptional silencing is also sensitive to *piwi* mutations suggests that RNAi may also mediate this transcriptional silencing. It has also been shown that RNAi can trigger both transcriptional and post-transcriptional silencing of transgenes in plants (Sijen et al, 2000).

In our system, the *trans*-silencing of *E1* and *E2* was lost or reduced when the chromosome 4 containing *P(lacW)* was translocated onto other chromosomes. This disruption suggests that this *trans*-silencing needs chromosome pairing and thus occurs at the transcriptional level. As mentioned above, RNAi can trigger both transcriptional and post-transcriptional silencing of transgenes. Therefore, the post-transcriptional silencing model of RNAi cannot apply to our *trans*-silencing system whereas the transcriptional one can. In our *trans*-silencing system, the length of the shared sequence between the *P(lacW)* of *w* mutant

alleles and that of the *Pci* allele on the other homolog can affect the extent of the *trans*-silencing of those *w* mutant alleles. It is possible that the length of the shared *P(lacW)* sequence between the transmitter sequence and the receiver sequence can affect the production of aberrant RNA, which, in turn, influences the degradation of homologous RNAs.

4.5.5 A combination of two models may also apply to the *trans*-silencing of *E1* and *E2* alleles

It is also possible that a combination of *trans*-heterochromatinization and RNAi is responsible for the *trans*-silencing (Ronsseray et al, 2003). The heterochromatinized nature of the transmitter sequence may produce aberrant RNAs or double-strand RNAs. Such RNAs confer a chromatin state to the local receiver sequence (Ronsseray et al, 2003).

It has been showed that RNA interference (RNAi) is required to establish silencing in heterochromatic regions in fission yeast (Volpe et al, 2002). In the fission yeast *Schizosaccharomyces pombe*, the *argonaute*, *dicer* and RNA-dependent RNA polymerase (*RdRP*) genes consist of part of RNA interference (RNAi) machinery. Volpe et al (2002) used *S. pombe* as the model system, deleted the *argonaute*, *dicer* and *RdRP* gene homologs, and examined how that could affect centromeric heterochromatin. The deletion results in transcriptional silencing of transgenes integrated at the centromere, loss of histone H3 lysine-9 methylation associated with the transgene integrated at the centromere or associated with centromeric heterochromatic repeats, and the aberrant accumulation of complementary transcripts from centromeric heterochromatic repeats. Eukaryotic heterochromatin is generally characterized by repeats and transposons and histone H3 lysine-9 methylation, and can influence gene expression. Volpe et al (2002) proposed that the formation and maintenance of centromeric heterochromatin is mediated by RNAi, which may be targeted by double-stranded RNA arising from centromeric repeats.

Recent work demonstrated that in *Drosophila* RNAi components (*aubergine*, *piwi* and *homeless*) are required for heterochromatic silencing of the *P* element *P[hsp26-pt, hsp70-w]* inserted in the pericentromeric heterochromatin (line 118E-10) or in the fourth chromosome (line 39C-12) (Pal-Bhadra et al, 2004). In *Drosophila*, the *piwi*, *aubergine* and *spindle-E* (*homeless*) genes encode RNAi components. When the *P* construct is inserted in a euchromatic domain, the insertion results in a uniformly red eye. However, insertions in the pericentric heterochromatin (in line 118E-10) or in the fourth chromosome (in line 39C-12) results in a variegating eye phenotype. Dominant suppressors of PEV can affect the variegating phenotype in the two lines, suggesting that it is heterochromatic silencing (Wallrath et al, 1995). Pal-Bhadra et al (2004) examined the impact of *piwi*, *aubergine*, and

homeless mutant alleles on the two variegating lines; they observed that the functions of all three loci are required for this heterochromatic silencing.

In *Drosophila*, histone H3 lysine-9 methylation (H3-mK9) and HP1 are known components of heterochromatin (Shaffer et al, 2002). Thus, Pal-Bhadra et al (2004) examined the impact of *piwi*, *aubergine*, and *homeless* mutant alleles on HP1 and H3-mK9 in the two variegating lines. They found that these mutations affect the distribution of HP1 but not the expression of HP1 and reduce histone H3-mK9 significantly. These changes are consistent with the loss of the heterochromatic silencing. Taken together, their results suggest that the RNAi system is required for proper localization of HP1, methylation of histone H3 at lysine 9, and thus the heterochromatic silencing.

In this study, the *trans*-silencing of *P(lacW)* by *E1* and *E2* alleles is suppressed by *Suppressors-of-variegation* (*Su(var)s*), which act generally to suppress PEV mutations (Grigliatti, 1991). This includes HP1, thereby confirming a role for heterochromatin in this *trans*-silencing (Martin-Morris et al, 1997). Therefore, it will be important to determine whether RNAi machinery is required for this *trans*-silencing.

4.5.6 *E2* allele is recessive lethal, whereas its excision derivatives are not

The *E2* allele is recessive lethal, however, *Pci* alone is not recessive lethal and the *E2* precise excision derivatives (that retain the *gypsy*) are also not recessive lethal. Only *E2*, with both the *gypsy* and *P(lacW)* insertions, is recessive lethal. Therefore, neither insertion by itself is sufficient to cause the recessive lethal phenotype of *E2*.

To map the location of the recessive lethal, flies containing *E2* allele can be crossed to flies containing the deficiency spanning the region where *P(lacW)* is inserted. If there is no *E2/deficiency* flies (with a variegated eye phenotype) in the progeny, then the recessive lethal is located in the region where *P(lacW)* is inserted in *E2*.

4.6 Future work

4.6.1 How to determine whether RNAi machinery is required for this *trans*-silencing:

Since *piwi*, *aubergine*, and *homeless* genes are components of RNAi machinery in *Drosophila*, the impact of mutant alleles of these three genes on this *trans*-silencing can indicate whether RNAi machinery is required for this *trans*-silencing. If RNAi machinery is involved in this *trans*-silencing, then this *trans*-silencing is RNAi mediated.

4.6.2 A genetic screen to search for more genes involved in this *trans*-silencing:

In addition, a genetic screen can be conducted to search for second site modifiers for this *trans*-silencing. The second site modifiers found in the screen can then be tested to determine whether they are classic PEV suppressors (*Su(var)s*), components of RNAi machinery, or genes of other functions.

If the modifiers are classic PEV suppressors, it confirms that heterochromatin plays a role in this *trans*-silencing. If the modifiers are components of RNAi machinery, it indicates that RNAi machinery is involved in this *trans*-silencing. If the modifiers are genes of other functions, these genes with newfound functions can shed some light on what mechanism is involved in this *trans*-silencing.

Chapter 5: References and Appendix

References

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Appendix

Appendix 1. The genotypes of the stocks used in this study

Genotype	Usage
<i>w</i> ; <i>Sb e Δ2-3 /TM3</i> ; <i>ci¹ ey¹</i> <i>w</i> ; <i>P(lacW)ci^{F1}</i> <i>w</i> ; <i>P(lacW)ci^{F2} / Dp(2;4)ey^D, ey^DAlp^{eyD}</i> <i>w</i> ; <i>dp</i> ; <i>e</i> ; <i>ci¹ ey¹</i> <i>w</i> ; <i>P(lacW)ci^{Dplac}</i>	These stocks were used in the Δ2-3 screen
<i>y w</i> ; <i>ci^D / M^{57g}</i> <i>w</i> ; <i>Dp(2;4)ey^D, ey^DAlp^{eyD} / Df(4)M101-62f</i>	These stocks were used to stock <i>w</i> ⁺ and <i>w</i> ⁻ mutant alleles
<i>ci^{Ce-2} / Dp(2,4)ey[D], Alp[eyD]; ey[D]</i> <i>ci^W</i> <i>ci^{57g}</i> <i>y w</i>	These stocks were used to test if <i>ci^{Ce-2}</i> , <i>ci^W</i> , <i>ci^{57g}</i> and + alleles can <i>trans</i> -silence <i>Pci</i> , respectively
<i>w</i> ; <i>Su(var)3-7^{9g} / TM6B; Pci / Pci</i>	This stock was used to test whether the mutation in <i>Su(var)3-7</i> can affect the strong <i>trans</i> -silencing of <i>Pci</i> by <i>w</i> ⁻ mutant alleles
<i>OregonR (+/+)</i>	This stock was used for polytene chromosome squash

Appendix 2. PCR reaction mix

1X recipe

3 μ l of 10X buffer (supplied with enzyme)
0.6 μ l of dNTP mix (10mM of each dNTP)
0.9 μ l of $MgCl_2$ (50mM, supplied with enzyme)
1.5 μ l of forward primer (10mM)
1.5 μ l of reverse primer (10mM)
21.35 μ l of dH_2O (sterile)
0.15 μ l of Taq DNA Polymerase

29 μ l volume

Appendix 3. The reaction mix for sequencing of PCR products

1X recipe

2 μ l of Big Dye premix
6 μ l of reaction buffer (200mM Tris-HCl pH9, 5 mM $MgCl_2$)
10 μ l of extracted DNA
2 μ l of primer (2.5 μ M)

20 μ l

Appendix 4. Random primer labeling reaction

1X recipe

33 μ l DNA (50-100ng) + dH_2O - Boil for 3-5 min
10 μ l 5X OLB (details as follows)
5 μ l ^{32}P -dCTP (10 mCi/ml at 3000 Ci/mmol)
2 μ l Klenow Gibco (1-9 U/ μ l)

50 μ l volume

After making 5xOLB as follows, I allocated 100 μ l into separate tubes. Add 2 μ l β -mercaptoethanol to each tube before use and keep in freezer.

5X OLB

100 μ l of 1M TRIS pH 7.5
12.5 μ l of 1M $MgCl_2$
1.25 μ l of dATP (100mM)
1.25 μ l of dGTP (100mM)

1.25 μ l of dTTP (100mM)
250 μ l of 2M HEPES pH 6.6
150 μ l of 90A₂₈₀ units of pd(N)₈
3.75 μ l of dH₂O
520 μ l

Appendix 5. Hybridization solution and wash solutions

HYBRIDIZATION SOLUTION

50mM Na₃PO₄ pH 7.2

6X SSC

1% SDS

WASH SOLUTIONS

WASH I

2X SSC

0.1% SDS

WASH II

0.2X SSC

0.1% SDS

WASH III

0.1X SSC

0.1% SDS